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Título de la tesis (español e inglés): Mejoras del proceso de elaboración de la aceituna de mesa con DOP *Aloreña de Málaga*: Análisis de Control de Peligros y Puntos Críticos en las industrias.

Improvements of the elaboration process of table olive with *Aloreña de Málaga* PDO: Hazard Analysis and Critical Control Points in the industries.

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TITULO: *MEJORAS DEL PROCESO DE ELABORACIÓN DE LA ACEITUNA DE MESA CON DOP ALORENA DE MÁLAGA: ANÁLISIS DE CONTROL DE PELIGROS Y PUNTOS CRÍTICOS EN LAS INDUSTRIAS*

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TESIS DOCTORAL

MEJORAS DEL PROCESO DE ELABORACIÓN DE LA ACEITUNA DE MESA CON DOP *ALOREÑA DE MÁLAGA:* ANÁLISIS DE CONTROL DE PELIGROS Y PUNTOS CRÍTICOS EN LAS INDUSTRIAS

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UNIVERSIDAD DE CÓRDOBA

DEPARTAMENTO DE BROMATOLOGÍA Y TECNOLOGÍA DE LOS ALIMENTOS

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CERTIFICAN:

Que el trabajo presentado por el Licenciado Miguel Ángel Ruiz Bellido con el título: **"Mejoras del proceso de elaboración de la aceituna de mesa con DOP *Aloreña de Málaga*: Análisis de Control de Peligros y Puntos Críticos en las industrias"** se ha desarrollado bajo nuestra dirección y tutela, durante los años 2014-2018, considerando que reúne los requisitos necesarios para optar al grado de Doctor por la Universidad de Córdoba.

Y para que así conste, expedimos el presente certificado en Córdoba, a 13 de Junio de 2018.

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TÍTULO DE LA TESIS: MEJORAS DEL PROCESO DE ELABORACIÓN DE LA ACEITUNA DE MESA CON *DOP ALOREÑA DE MÁLAGA*: ANÁLISIS DE CONTROL DE PELIGROS Y PUNTOS CRÍTICOS EN LA INDUSTRIAS

DOCTORANDO: D. Miguel Angel Ruiz Bellido

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La presente Tesis Doctoral se enmarca en el proyecto motriz de la Junta de Andalucía (convocatoria 2011: AGR-7755) titulado "Control de las poblaciones de enterobacterias en envasados de aceitunas de mesa con DOP *Aloreña de Málaga*" (acrónimo PrediAlo) y del Programa de Formación de Doctores en Empresa (convocatoria 2013) del Campus de Excelencia Internacional Agroalimentario ceiA3 de las Universidades de Córdoba, Cádiz, Huelva, Almería y Jaén. A través de un convenio firmado entre el ceiA3 y la Asociación de Aderezadores de Aceituna de Mesa de la Comarca Natural del Valle del Guadalhorce y Sierra de las Nieves, se reguló la figura del investigador senior en la empresa (el presente doctorando), y gracias a los recursos propios del proyecto PrediAlo con presencia de investigadores del Instituto de la Grasa (CSIC: grupo PAIDI AGR-125) y la Universidad de Córdoba (grupo HIBRO: PAIDI AGR-170), se pudo disponer de todo el material fungible y equipamiento necesario para la realización de las actividades experimentales. De este modo, todo el trabajo de laboratorio ha sido llevado a cabo íntegramente en las instalaciones del Departamento de Biotecnología de Alimentos del Instituto de la Grasa (CSIC, Sevilla) y en el Departamento de Bromatología y Tecnología de los Alimentos de la Universidad de Córdoba (Grupo HIBRO). Por otro lado, la recogida de muestras ha sido llevada a cabo en diferentes agroindustrias de la provincia de Málaga que forman parte de la Asociación de Aderezadores y del Consejo Regulador de la DOP *Aloreña de Málaga*. Se trata por tanto de un claro ejemplo de investigación aplicada al sector industrial y cuya finalidad es la formación en el tejido productivo de personas cualificadas y de alto

rigor científico que sean capaces de llevar a cabo actividades de I+D+i de forma continuada.

La aceituna de mesa con DOP *Aloreña de Málaga* fue la primera aceituna de mesa de España en obtener la DOP por orden de 26 de octubre del 2009 de la Consejería de Agricultura y Pesca de la Junta de Andalucía (BOJA nº 215, 2009). Este reconocimiento fue posteriormente homologado por la Unión Europea (DOUE 2012). La presente Tesis Doctoral surge de la necesidad por parte del sector de mejorar y modernizar el proceso de transformación de este tipo de aceituna para responder a las actuales demandas del mercado (estandarización de la producción, productos con un menor contenido en sodio y con mayores controles de calidad y seguridad). De este modo se continúa en la senda de los estudios iniciados por el grupo de investigación PAIDI AGR-125 del Instituto de la Grasa y que fueron presentados en el 2007 por el Dr. Francisco Noé Arroyo López (co-director de Tesis) mediante la defensa de la Tesis Doctoral titulada "Conservación y envasado de aceitunas de mesa aliñadas Manzanilla-Aloreña: Diseño de modelos matemáticos para el crecimiento e inhibición de las poblaciones de microorganismos". En dicha Tesis se inició un proceso de caracterización físico-química y microbiológica del proceso de transformación. Una década después, y a tenor de la evolución en las tendencias de consumo y las demandas de la gran distribución en lo que respecta a la seguridad alimentaria y certificaciones de calidad, se hizo necesario profundizar aún más en el conocimiento de los procesos, aplicando nuevas metodologías y enfoques (ómicas y técnicas estadísticas) y valorando la utilización de los nuevos datos obtenidos en materia de calidad y seguridad alimentaria.

Los resultados que se incluyen en la Tesis se presentan como un compendio de publicaciones de acuerdo con la normativa reguladora de los estudios de doctorado en la Universidad de Córdoba, y se ha dividido en las siguientes secciones:

> Primera sección. Incluye estudios encaminados a modificar el proceso de elaboración de aceitunas de mesa con DOP Aloreña de Málaga para reducir el contenido en sodio y mejorar la calidad de los frutos, así como la introducción de nuevas herramientas matemáticas (*functional data analysis*) y técnicas ómicas (metagenómica) para el estudio de los procesos. En esta sección se incluyen 5 trabajos científicos, todos ellos publicados en revistas con alto índice de impacto: International Journal of Food Microbiology 2016: http://dx.doi.org/10.1016/j.ijfoodmicro.2016.08.031; Data in Brief 2016: http://dx.doi.org/10.1016/j.ijfoodmicro.2016.08.031; International Journal of Food

Microbiology 2016: http://dx.doi.org/10.1016/j.ijfoodmicro.2016.07.014; PLOS ONE 2016: DOI:10.1371/journal.pone.0163135; Frontiers in Microbiology 2017: doi: 10.3389/fmicb.2017.02209).

> Segunda sección. Incluye el mayor estudio realizado en el sector hasta la fecha para determinar el grado de calidad y seguridad alimentaria del proceso de transformación, de cara a la implantación de medidas encaminadas a la mejora de las condiciones higiénico-sanitarias y sistemas APPCC. Fruto de este estudio, se ha procedido a proteger intelectualmente mediante secreto industrial (3731/2016) un procedimiento científico para la gestión de la calidad y seguridad alimentaria en las empresas que elaboran aceitunas de mesa y que está siendo explotado por la EBT TAFIQs in FOODS. En esta sección se incluyen 1 trabajo científico: Frontiers in Microbiology 2017: doi: 10.3389/fmicb.2017.02326.

La Tesis Doctoral se ha llevado a cabo desde un enfoque multidisciplinar a lo largo de cuatro años de estudio (2013-2017), utilizando técnicas clásicas de microbiología, biología molecular, herramientas ómicas, microbiología predictiva, química analítica y análisis sensorial, a lo que hay que sumar un considerable número de visitas a las empresas para la obtención de muestras para analíticas físico-químicas y microbiológicas y mejora y de sistemas de autocontrol en las industrias. Los resultados generados en el presente estudio constituyen una fuente de información relevante para la comunidad científica, el sector industrial y las autoridades sanitarias en materia de calidad y seguridad alimentaria, siendo la base a su vez para el desarrollo de futuros trabajos científicos

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Sevilla, 13 de junio de 2018

Firma del/de los director/es

Fdo.: Dr. Antonio Valero Díaz **Fdo.: Dr. Francisco Noé Arroyo López**

Fdo.: Dr. Eduardo Medina Pradas

AGRADECIMIENTOS

Llegado este momento, por donde comenzar y, como todas las cosas, por el principio. Aunque parezca una broma, a la vez que muy típico en esta tierra de la que tan orgullosos nos sentimos, toda esta aventura comenzó "echando un Gin-Tonic" una tarde en el Pub Paraíso de la localidad de Alozaina (Málaga) y tras una reunión de trabajo con los responsables de las cooperativas que integran el Grupo Aloreña, SCA, una cooperativa de 2º grado compuesta por las cooperativas de Alora, Casarabonela, Alozaina, Tolox y Guaro, y con un objetivo bien marcado, esto es, la comercialización de las aceitunas con DOP *Aloreña de Málaga* envasadas y listas para consumir. Pues bien, tras esa reunión, el actual gerente de la cooperativa de Alozaina (Copusan, SCA), D. Daniel Arias y un servidor nos vamos a despejarnos un poco y continuar hablando de trabajo, pero en otro ambiente más distendido y, durante la conversación, surge la posibilidad de participar en un proyecto de "Doctores en Empresa" impulsado desde el Instituto de la Grasa y en la persona de uno de mis directores de tesis, D. Francisco Noé Arroyo junto con la Asociación de Aderezadores, impulsora de la DOP *Aloreña de Málaga*. Ahí comienza la aventura y, en cierta medida, volver al ámbito de la investigación, aunque esta vez, desde una óptica radicalmente distinta a la convencional ya que, tal y como se lo trasladé al equipo técnico del Campus ceiA3 en relación con el programa "Doctores en Empresa", la formación de doctores dentro de las empresas es altamente interesante para el desarrollo de una nueva visión sobre la configuración presente y futura de la microempresa agroalimentaria que, en muchos casos y sin saberlo, la necesita y no puede permitirse en demasiadas ocasiones, la innovación de forma imperiosa como elemento de competitividad para continuar viva en un entorno empresarial cada vez más globalizado y tensionado en el que, el principal factor de competitividad es el precio.

Si bien ese día en el que me embarqué en este proyecto, siempre he sabido y palpado el respaldo inmenso e incondicional que he tenido en mi familia, en mis padres que se han "dejado el pellejo" para que nosotros, mi hermano y yo, podamos perseguir nuestros sueños y, que nunca han tenido un "no" a la hora de echarnos una mano en lo que hayamos necesitado. Echándoles la vista encima, se les ve que tienen un corazón inmenso y desprenden la satisfacción de haber conseguido su sueño, tan simple y tan duro como que sus hijos tuviesen más formación de la que ellos habían tenido.

Al mismo nivel, y con quién he tenido la fortuna de compartir cada segundo de mi vida desde hace casi 15 años, mi mujer "Laly – Candela – Cande", una persona con un bondad inmensa, un corazón como una plaza de toros, que me ha permitido entregarme a este y otros proyectos con los que perseguir ese sueño, que no es otro, que el ser un buen espejo en el que se puedan reflejar y sentirse orgullosos mis dos hijos, mis dos soles, Miguel y María, mi mayor motivación para crecer como persona, sabiendo que el mejor legado que le podemos dejar a nuestros hijos es, ese sentimiento de orgullo cuando piensen "mis padres hicieron todo lo que estuvo en sus manos".

También me gustaría dejar sobre este documento los nombres de otras personas que han contribuido muy activamente a este momento, comenzando por mis directores de Tesis, los Drs. Antonio Valero Díaz, Francisco Noé Arroyo López y Eduardo Medina Pradas. Gente de inmensa valía personal y profesional con una capacidad de entrega digna de elogiar y difundir a los cuatro vientos. Es díficil de comprender como en la sociedad de la información se entierra hechos y personas tan relevantes como este grupo de investigación que tanto y tan bueno está haciendo en pos de la aceituna de mesa en Andalucía y fuera de ella. Seguro que ese reconocimiento les llegará en algún momento ya que, por desgracia, en demasiadas ocasiones no se le otorga y reconoce la importancia y el valor que se debe y su gran contribución a la sociedad, siempre desde los centros públicos de formación e investigación.

Una mención especial para mi "partenaire" en este proyecto PrediAlo, la recién nombrada Dra. Verónica Romero Gil, una compañera de viaje ideal, capaz de implicarse "hasta las cejas", encomiable su proactividad y capacidad de entrega, discreta y humilde. Una bellísima profesional y mejor persona.

Junto a ellos cuatro, el resto de socios/as que forma parte del proyecto TAFIQs in FOODs – OLEICA, D. Francisco Rodriguez, D. Rufino Jiménez, Dña. Beatriz Calero, D. Antonio Benitez, D. Daniel Jiménez, D. Alfredo Rivero y D. Juan A. Berrocal como representante del Consejo Regulador. Un conjunto de amigos/as y socios con los que está siendo un placer compartir todos y cada uno de los momentos, en los que todas/os estamos aprendiendo mucho sobre cómo debemos compatibilizar las distintas facetas que, según parece, va a conllevar la labor investigadora en el presente-futuro. Mientras que se perfilan los últimos detalles de esta tesis, estamos preparando la entrada de otros dos socios/as más ya que

está en el espíritu de este proyecto empresarial funcionar a modo de cooperativa y crear un equipo multidisciplinar y máximamente solvente. Estos dos nuevos/as compañeras/os serán Dña. Eva Ramirez y D. Manuel Ruiz.

Para nada me gustaría dejar por escribir y reconocer la importancia que han tendido durante estos años 3 grandes personas que, por delante su faceta de magníficos investigadores/as, tienen y desprenden una enorme valía humana, ellos son los Drs. D. Antonio Garrido, D. Pedro García García y Dña. Concepción Romero Barranco que siempre me han brindado un trato cercano y comprensivo, dado lo parcial del tiempo que le he tenido que dedicar a esta etapa de mi vida, y me han hecho sentir dentro de la institución "como en casa".

Además, y para finalizar, no quisiera dejar por escribir y recordar en este documento los nombres de tres personas y magníficos profesionales, tanto como la copa de un pino, en el ámbito del fomento del emprendimiento desde lo público. Estas personas son D. Gabriel J. Clavijo Sánchez en la responsabilidad de Director Provincial de AEFPA, Dña. Aurora García Cabrera en la responsabilidad de Responsable de Zona y a Dña. Gema Maza Gómez en la responsabilidad de administrativo del CADE de Guaro que, además de ser soporte indirecto e imprescindible para la consecución de los objetivos marcados en la presente Tesis, marcados o no desde el ámbito oficial, pero siempre diseñados para el beneficio de los usuarios/as y la ciudadanía en general a la que nos debemos.

En el ámbito de la empresa, me gustaría resaltar la importancia que han tenido las entidades participantes, esto es, Copusan, SCA – Tolox Agrícola, SCA – Aceitunas Roldán, SL. – Manzanilla Aloreña, SCA – Aceitunas y Encurtidos Bravo - y la agrupación de cooperativas SCA Sierra de las Nieves – Grupo Aloreña de 2º Grado. Estas entidades no serían nada sin sus responsables, a saber, D. Daniel Arias Meneses, Dña. María Vázquez Mesa, D. Manuel Roldán, D. Francisco Pérez García y la Familia Bravo, todos ellos, grandes profesionales y visionarios dentro de sus entidades que han sabido ver la importancia de la mejora de procesos, de la innovación y el desarrollo de nuevos productos capaces de dar respuesta a las presentes y futuras demandas del mercado, además de participar en una organización como es el Grupo Aloreña de 2º Grado, SCA para darle la adecuada dimensión empresarial al importante valor que ya tiene la aceituna con DOP *Aloreña de Málaga*. No

puede faltar el merecido reconocimiento al staff técnico del Consejo Regulador en las personas de Dña. Margarita Jiménez y Dña. María José Guerrero además de la figura de su presidente D. Juan A. Berrocal, todas/os ellas/os volcados con un proyecto y un producto hecho durante años solo para “creyentes incondicionales”.

Y por último, pero no menos importante, a las compañeras y compañeros que han hecho posible, desde El Molino de Guaro, SCA, donde nace todo allá por finales del 2008 y que, sin su apoyo y complicidad no hubiese sido posible avanzar en este reto.

A todos y cada uno de ellos, gracias.

Gracias a mis padres, dos viejitos a los que quiero con todo mi alma

“La fuerza no viene de la capacidad corporal, sino de la voluntad del alma”

Gandhi

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RESUMEN

La *Aloreña de Málaga* es una variedad de aceituna de mesa autóctona del Valle del Guadalhorce y la Sierra de las Nieves, con una demarcación muy concreta (19 municipios) siendo muy apreciada dentro y fuera de la provincia de Málaga. Fue la primera aceituna de mesa en España en obtener el sello de calidad de DOP (BOJA nº215, 2009). El volumen de producción de este tipo de especialidad de aceitunas de mesa es de aproximadamente 3.500-4.000 ton/año según datos estimados por el Ministerio de Agricultura (http://www.aica.gob.es/) y contrastados con las declaraciones de los industriales, realizadas por el Consejo Regulador. Por lo tanto, la producción no es muy elevada comparada con otros tipos de elaboraciones como son las aceitunas verdes estilo español o sevillano. Sin embargo, su elaboración aún hoy en día se sigue realizando de manera muy artesanal (un aspecto este altamente valorado por los consumidores), y es llevado a cabo en industrias de pequeño tamaño (<20 empleados) las cuales tienen un gran interés por optimizar y mejorar los procesos de transformación de cara a la obtención de un producto con los mayores estándares de calidad y seguridad, tal y como se exige por parte de la gran distribución.

La presente Tesis Doctoral está centrada en suministrar una mayor información de todos los procesos que ocurren durante la transformación de este tipo de elaboración de aceitunas de mesa en la industria, así como del efecto de la introducción de nuevas modificaciones sobre la calidad y seguridad alimentaria del producto final. De este modo, la Tesis Doctoral está dividida en dos secciones. En la primera de ellas se aplican nuevas metodologías como la metagenómica y el modelado estadístico con el objetivo de obtener un mayor conocimiento de cómo diferentes modificaciones (reducción del contenido en cloruro de sódico, reposo de las aceitunas y aplicación de un tratamiento térmico a los frutos) afectan al proceso. En la segunda se pretende valorar los sistemas de análisis de peligros y puntos de control críticos de distintas industrias del sector en diferentes campañas y determinar el grado de cumplimiento higiénico-sanitario a través de analíticas físico-químicas y microbiológicas a lo largo de la cadena de elaboración. La información obtenida permitirá el desarrollo posterior de un procedimiento científico basado en modelos matemáticos y diferentes tipos de ponderaciones para la gestión de la calidad y seguridad alimentaria en las industrias.

Para el estudio del efecto de la eliminación del cloruro de sodio y la adición de un período de reposo de los frutos en el proceso de la fermentación y posterior conservación de las aceitunas, se recolectaron 1.500 kg de frutos y se colocaron en bombonas con distintas

salmueras de conservación variando las concentraciones de ácido acético, cítrico y sal, directamente o con un período de reposo de las aceitunas a temperatura ambiente (72 h, 25ºC). Posteriormente, se monitorizaron los principales parámetros físico-químicos y microbiológicos durante un periodo de 1 año. La presencia de una alta cantidad de ácido acético, junto con la ausencia de sal, no afectó negativamente a la evolución de los principales parámetros físico-químicos, aunque existieron diferencias según el tratamiento ensayado. El proceso estuvo caracterizado por la rápida inhibición de las poblaciones de enterobacterias, así como por el crecimiento de bacterias lácticas (solo al final del proceso) y especialmente levaduras, que estuvieron presentes durante todo el tiempo alcanzando niveles poblaciones de 6 $\log_{10}$ UFC/mL. La aplicación por primera vez en la microbiología de alimentos de un modelo matemático de análisis de datos funcionales programado en R permitió realizar una sofisticada comparación de los diferentes tratamientos ensayados, obteniendo también nueva información sobre la velocidad y aceleración de los cambios que ocurren, medias, máximos y mínimos obtenidos. El ajuste mostró que el desarrollo de las poblaciones de levaduras fue muy parecido entre los diferentes tratamientos ensayados, aunque en algunos tiempos a lo largo del proceso fermentativo existieron diferencias significativas entre los mismos. Sin embargo, se encontraron mayores diferencias para la evolución del pH, sobre todo durante los primeros 3 meses del proceso, estabilizándose después todos los tratamientos en torno a un valor de pH de 4,0 unidades aproximadamente.

Para la aplicación de técnicas de secuenciación masiva en el estudio del proceso de fermentación y conservación de la aceituna *Aloreña de Málaga*, se monitorizaron 4 bombonas de fermentación (aproximadamente 500 kg de aceitunas) en la industria por un periodo de tiempo de 4 meses llevándose a cabo un análisis físico-químico y microbiológico (enterobacterias, bacterias lácticas y levaduras). Desde el punto de vista físico-químico, la fermentación se completó de la manera habitual, siendo las levaduras los únicos microorganismos que se desarrollaron durante el proceso fermentativo sin la aparición de enterobacterias o bacterias lácticas. Durante los 4 meses de fermentación, se tomaron muestras tanto de salmueras como de los frutos para su análisis metagenómico con el objetivo de estudiar la ecología de las poblaciones bacterianas (16S) y fúngicas (ITS). Esta metodología permitió detectar con relativa frecuencia la presencia de géneros que anteriormente no habían sido descritos en el producto como son *Celerinatantimonas*, *Pseudomonas* y *Propionibacterium* entre las bacterias, y *Penicillium*, *Pichia* y

Zygotorulaspora entre las levaduras y hongos. No se detectó la presencia de ninguna especie potencialmente patógena, lo que garantiza la seguridad microbiológica de las aceitunas fermentadas. Por lo tanto, la aplicación de esta técnica independiente de cultivo ha permitido ampliar la información de especies bacterianas que no habían podido ser detectadas por las metodologías clásicas. Queda por determinar en futuros estudios el papel que pueden tener estas nuevas especies en la calidad del producto final.

Para el estudio de la aplicación de tratamientos térmicos a los frutos y su efecto sobre la fermentación y las características organolépticas de los frutos, se tomaron 100 kg aproximadamente de frutos partidos (destinados a la elaboración de aceitunas tradicionales) y enteros (destinados a la elaboración de aceitunas curadas). Estos frutos fueron sometidos a un tratamiento térmico que consistió en sumergir los frutos en agua a una temperatura de 60ºC durante 5 minutos antes de su colocación en salmuera. Posteriormente, las fermentaciones fueron monitorizadas por un periodo de tiempo que superó los 5 meses. La realización de este tratamiento térmico de las aceitunas previo a su colocación en salmuera favoreció la fermentación láctica, con un mayor consumo de azúcares, una mayor producción de acidez libre y bajada de pH que garantiza la estabilidad de los frutos. En las aceitunas tradicionales tratadas térmicamente, los niveles poblacionales de las bacterias lácticas alcanzaron los 5 $\log_{10}$ UFC/mL, mientras que en las salmueras sin tratamiento térmico estos microorganismos no se detectaron. La mejora de la coloración de los frutos (luminosidad, color verde, etc.) fue también muy apreciable en los frutos que fueron sometidos a tratamiento térmico, tanto durante la fase de fermentación como en el posterior envasado, presentando una mayor luminosidad y tonalidad verde. La gran ventaja de la realización del tratamiento con calor se confirmó después también en la mayor estabilidad y aceptabilidad de los frutos una vez que fueron envasados. De este modo, a los 41 días de envasado, el producto que tenía una mayor frecuencia de catadores que consumirían el producto y que comprarían el envase se correspondió con el producto tradicional tratado térmicamente, variando sus porcentajes de aceptación escasamente entre los 4 y 41 días de envasado, lo cual no ocurrió para el resto de los tratamientos ensayados. Finalmente, el análisis metagenómico realizado al final de la fermentación a todos los tipos de elaboraciones y entre frutos tratados térmicamente y no tratados, reveló que los género *Lactobacillus, Pediococcus* y *Celerinatantimonas* estuvieron presentes en todos los tipos de elaboración, tanto en fruto como en salmuera, y que la prevalencia de *Lactobacillus* fue significativamente más elevada en los frutos tratados

térmicamente, lo que confirma que la fermentación láctica fue mejorada por la aplicación del calor.

Finalmente, en la presente Tesis Doctoral se han visitado 3 empresas referentes del sector de la aceituna *Aloreña de Málaga* en 3 campañas sucesivas de elaboración (13/14, 14/15, y 15/16) obteniéndose muestras de control ambiental, superficie de las maquinarias y operarios, frutos, aliños, líquidos de gobierno y aguas de aljibe para la realización de analíticas de tipo microbiológico y físico-químicas (>650). De este modo, se han estudiado los niveles poblacionales de aerobios mesófilos, levaduras, bacterias lácticas, enterobacterias, esporulados, presencia de *Listeria*, *Salmonella*, *Clostridium* y *Staphylococcus* coagulasa positivos. En base a los resultados obtenidos, se generó un modelo matemático que estimó el grado de cumplimiento higiénico-sanitario del proceso de elaboración de este tipo de aceituna de mesa mediante un sistema de ponderación (basado en la legislación presente y opiniones de expertos) y modelo probabilístico de gestión del riesgo. La contribución relativa de cada fase a la calidad y seguridad del producto final fue evaluada y ponderada por un panel de expertos (n=25) del ámbito científico y del sector de la *Aloreña de Málaga*. No se ha detectado en ninguna empresa la presencia de especies patógenas que puedan suponer un riesgo para los consumidores. La etapa de aliño ha resultado ser la más crítica dentro del proceso, ya que las hierbas aromáticas y diversos ingredientes que se utilizan en el mismo presentan una elevada carga microbiana que se añade directamente a los envasados sin ningún tipo de tratamiento previo. También es recomendable reducir el número de microorganismos en el ambiente y un mayor control de las aguas destinadas al consumo humano. Se observó asimismo que el grado de cumplimiento higiénico-sanitario determinado por el sistema de evaluación desarrollado aumenta conforme se acercan las etapas finales del proceso, las cuales resultan ser las más seguras. Con toda la información obtenida, se ha desarrollado un protocolo y procedimiento científico que puede ser utilizado por las empresas del sector para estandarizar sus procesos, valorar cuantitativamente el grado de cumplimiento higiénico-sanitario de las diferentes etapas de producción y predecir los efectos de la introducción de medidas correctoras sobre la calidad y seguridad de los productos finales.

SUMMARY

The *Aloreña de Málaga* table olive is an autochthonous variety from Guadalhorce Valley and Sierra de las Nieves region, with a confined demarcation (19 municipalities) being well appreciated inside and outside Malaga province. It has been the first table olive with Protected Designation of Origin (PDO) in Spain (BOJA No. 215, 2009). The production volume of this table olive speciality is below 3,500-4,000 ton according to estimated records of the Ministry of Agriculture ((http://www.aica.gob.es/) and further verified with the interviews performed by the Regulatory Council to industrials. Thus, production is limited in comparison to other elaborations such as the Spanish table olive style. However, its elaboration is still very artisanal (which is highly appreciated by consumers), being carried out by SMEs with less than 20 operators. These industries have a great interest in optimizing and improving the transformation processes in order to obtain a valorised product with high quality and safety standards, which is currently requested by the large distribution chain.

This Doctoral Thesis is focused on providing additional knowledge about the transformation processes of *Aloreña de Málaga* table olives as well as to evaluate the effect of new changes on quality and safety of final products. In this sense, the Doctoral Thesis is divided into two sections. In the first section, novel methodologies such as metagenomics and statistical modelling are applied with the aim to gain knowledge about how different modifications (reduction of sodium chloride, inclusion of resting periods to table olives, and application of heat treatments) can affect to the elaboration process. In the second section, the hazard analysis and critical control points from various industries were evaluated in different campaigns in order to determine the fulfilment of hygienic-sanitary conditions through the performance of physicochemical and microbiological analyses along the production chain. Information generated will allow the further development of a scientific procedure based on advanced mathematical models and different weighing values to improve food quality and safety management in industries.

To study the effect of sodium chloride reduction and the addition of a resting period of fruits during fermentation and posterior table olive preservation, 1,500 kg of olive fruits were collected and placed in containers with different preservation brines and varying concentrations of acetic acid, citric acid and salt, either directly or with a resting period of table olives at room temperature (72 h, 25ºC). Afterwards, the main physicochemical and microbiological parameters were monitored during 1 year. The presence of a high amount of

acetic acid together with the absence of sodium chloride did not negatively affect the evolution of the main physicochemical parameters, though differences existed according to the assayed treatments. The elaboration process was marked by a rapid inhibition of *Enterobacteriaceae* population, as well as by a growth of lactic acid bacteria (just at the end of the process) and specially, by the yeasts growth, which were continuously present reaching concentration levels up to 6 $\log_{10}$ CFU/mL. One of the first applications in food microbiology of a functional data analysis model programmed in R was presented. The model allowed a smart comparison of the different assayed treatments, providing additional information on the rate and acceleration of changes in the elaboration process through the calculation of means, maximal and minimal values. Model fitting showed that yeasts population was very similar among the evaluated treatments, though significant differences existed for some fermentation treatment times. Nevertheless, larger differences were found for pH evolution, specially during the first 3- month process, being then stabilized after all treatments until a pH of 4.0 units approximately.

Next Generation Sequencing techniques were subsequently applied to study fermentation and preservation processes of *Aloreña de Málaga* table olives. To do this, four fermentation containers were monitored (approximately 500 kg of table olives) at the food industry during a 4-month period by performing physicochemical and microbiological (*Enterobacteriaceae*, lactic acid bacteria and yeasts) analyses. From a microbiological point of view, fermentation took place normally, being yeasts the only population group showing growth while lactic acid bacteria and *Enterobacteriaceae* were not present during fermentation. During the 4-month fermentation period metagenomic analysis of brines and fruit samples were carried out with the aim of studying the ecology of bacterial (16S) and fungal populations (ITS). This methodology allowed detecting with relative frequency bacteria genera not being previously described in this product such as *Celerinatantimonas*, *Pseudomonas* and *Propionibacterium* among the bacterial groups, and *Penicillium*, *Pichia* and *Zygotorulaspora* among yeasts and moulds. There was not any pathogenic microorganism in the fermented table olives studied by what microbial food safety was ensured. Therefore, the application of this culture independent technique has extended the microbial spectrum of bacterial species not being previously detected by conventional culture dependent techniques. The potential role of these microbial groups on the final product quality is still to be determined.

One-hundred kg. of cracked olives (destined to the elaboration of traditional table olives) and whole olives (destined to the elaboration of cured table olives) were collected to study the effect of a previous heat treatments on the fermentation process of olive fruits together with their organoleptical characteristics. These fruits were submitted to a heat treatment which consisted on a water immersion at 60ºC for 5 min before placing them in brine. Afterwards, fermentations were monitored during a study period longer than 5 months. Performance of this heat treatment in table olives previously to their transferring to brines enhanced lactic acid fermentation, with a higher sugars consumption, free acidity production and pH drop, which guaranteed the stability of olive fruits. In the heat-treated fermented table olives, population levels of lactic acid bacteria reached 5 $\log_{10}$ CFU/mL, while in those brines without a heat treatment, this microbial group was not detected. Improvement of fruit coloration (luminosity, green colour etc.) was observed in those fruits undergoing a heat treatment during fermentation and packaging steps. The great advantage of the applied heat treatment was subsequently confirmed by the improvement in the stability and acceptability of table olives once packaged. Indeed, 41 days after packaging, the products having the highest proportion of olive tasters who would consume them and would purchase the packaging, corresponded to the traditional heat-treated table olives. The acceptability percentages sparsely varied between 4 and 41 after packaging, which did not occur with the rest of assayed treatments. Finally, the metagenomic analysis performed at the end of the fermentation period to all elaboration types, and between treated and non-treated olive fruits revealed that the bacteria genera *Lactobacillus, Pediococcus* and *Celerinatantimonas* were present in all elaborations in both fruits and brines, and that prevalence of *Lactobacillus* was significantly higher in heat-treated olive fruits, which confirmed the improvement of lactic acid fermentation by the heat treatment applied.

Finally, in the present Doctoral Thesis three representative companies of the *Aloreña de Málaga* table olive sector were visited during three consecutive campaigns (13/14, 14/15, y 15/16). Physicochemical and microbiological analyses (>650) were performed corresponding to environmental control samples, equipment surfaces, food handlers, olive fruits, olive dressings, brines, and potable water. In this way, population levels of mesophilic bacteria, yeasts, lactic acid bacteria, sporulated bacteria, together with presence of *Listeria*, *Salmonella*, *Clostridium* and coagulase positive *Staphylococci*, were analysed. Based on the obtained results, a mathematical model was developed to estimate a Performance Hygiene and Safety

Score (PHSS) of the elaboration process of this table olive type by means of a weighing system (based on current legislation applied and experts opinions) together with a probabilistic decision-making model. The relative contributions of each processing step to final product quality and safety were evaluated and weighted by a scientific expert panel (n=25) together with industrials from the *Aloreña de Málaga* table olive sector. There was not any food company having presence foodborne pathogens during the whole study period. Addition of olive dressings has resulted to be most critical processing step, since aromatic herbs and diverse ingredients used presented a high microbial load which is directly added to the packaged products without a posterior heat treatment. It is also recommended to reduce the environmental contamination as well as a more intensive control of the potable water destined to human consumption. Likewise, it was observed that the PHSS values determined by the developed mathematical model increased according to the final processing steps, being the safest ones. With the information obtained, a protocol and scientific procedure was performed, which can be used by industrials in order to standardize their elaboration processes, to quantitatively assess the degree of fulfilment of hygienic-sanitary conditions of the different production steps and to predict the effect of corrective measures on final products quality and safety.

LISTADO DE ABREVIATURAS Y SÍMBOLOS

ADN	Ácido Dexoxirribonucleico
ADF	Análisis de Datos Funcionales
AMT	Aerobios Mesófilos Totales
APPCC	Análisis de Peligros y Puntos de Control Críticos
ARN	Ácido Ribonucleico
ARPCC	Análisis de Riesgos y Puntos de Control Críticos
a_w	Actividad del agua
BAL	Bacterias Ácido Lácticas
BPF	Buenas Prácticas de Fabricación
BOJA	Boletín Oficial de la Junta Andalucía
DGGE	Electroforesis en gel con gradiente desnaturalizante
DOP	Denominación de Origen Protegida
EBT	Empresa de Base Tecnológica
g	gramos
h	horas
ha	hectáreas
HyEDA	Forma dialdehídica decarboximetilada del ácido elenólico unido a hidroxitirosol
kg	kilogramos
L	Litros
mill	millones
mL	mililitros
NGS	Next Generation Sequencing
OTU	Unidad Taxonómica Operacional
PCCs	Puntos de control crítico
PCR	Polymerase chain reaction
PPO	Polifenol Oxidasa
RAPD	Random amplified polymorphic DNA
REP	Repetitive element palindromic
SGSA	Sistemas de Gestión de la Seguridad Alimentaria
ton	toneladas
UFC	Unidades Formadoras de Colonias

MARCO DE LA TESIS DOCTORAL

La presente Tesis Doctoral se enmarca dentro del proyecto motriz de la Junta de Andalucía (convocatoria 2011: AGR-7755) titulado "*Control de las poblaciones de enterobacterias en envasados de aceitunas de mesa con DOP Aloreña de Málaga*" (acrónimo PrediAlo) y del Programa de Formación de Doctores en Empresa (convocatoria 2013) del Campus de Excelencia Internacional Agroalimentario *ceia3* de las Universidades de Córdoba, Cádiz, Huelva, Almería y Jaén. A través de un convenio firmado entre el *ceia3* y la Asociación de Aderezadores de Aceituna de Mesa de la Comarca Natural del Valle del Guadalhorce y Sierra de las Nieves, se reguló la figura del investigador senior en la empresa (el presente doctorando), y gracias a los recursos propios del proyecto PrediAlo con presencia de investigadores del Instituto de la Grasa (CSIC: grupo PAIDI AGR-125) y la Universidad de Córdoba (grupo HIBRO: PAIDI AGR-170), se pudo disponer de todo el material fungible y equipamiento necesario para la realización de las actividades experimentales. De este modo, todo el trabajo de laboratorio ha sido llevado a cabo íntegramente en las instalaciones del Departamento de Biotecnología de Alimentos del Instituto de la Grasa (CSIC, Sevilla) y en el Departamento de Bromatología y Tecnología de los Alimentos de la Universidad de Córdoba (Grupo HIBRO). Por otro lado, la recogida de muestras ha sido llevada a cabo en diferentes agroindustrias de la provincia de Málaga que forman parte de la Asociación de Aderezadores y del Consejo Regulador de la DOP *Aloreña de Málaga*. Se trata por tanto de un claro ejemplo de investigación aplicada al sector industrial y cuya finalidad es la formación en el tejido productivo de personas cualificadas y de alto rigor científico que sean capaces de llevar a cabo actividades de I+D+i de forma continuada.

La aceituna de mesa con DOP *Aloreña de Málaga* fue la primera aceituna de mesa de España en obtener la DOP por orden de 26 de octubre del 2009 de la Consejería de Agricultura y Pesca de la Junta de Andalucía (BOJA nº 215, 2009). Este reconocimiento fue posteriormente homologado por la Unión Europea (DOUE 2012). La presente Tesis Doctoral surge de la necesidad por parte del sector de mejorar y modernizar el proceso de transformación de este tipo de aceituna para responder a las actuales demandas del mercado (productos con un menor contenido en sodio y con mayores controles de calidad y seguridad). De este modo se continúa en la senda de los estudios iniciados por el grupo de investigación PAIDI AGR-125 del Instituto de la Grasa y que fueron presentados en el 2007 por el Dr. Francisco Noé Arroyo López (co-director de Tesis) mediante la defensa de la Tesis Doctoral titulada "*Conservación y envasado de aceitunas de mesa aliñadas Manzanilla-Aloreña:*

Diseño de modelos matemáticos para el crecimiento e inhibición de las poblaciones de microorganismos". En dicha Tesis se inició un proceso de caracterización físico-química y microbiológica del proceso de transformación. Una década después, y a tenor de la evolución en las tendencias de consumo y las demandas de la gran distribución en lo que respecta a la seguridad alimentaria y certificaciones de calidad, se hizo necesario profundizar aún más en el conocimiento de los procesos, aplicando nuevas metodologías y enfoques (ómicas y técnicas estadísticas) y valorando la utilización de los nuevos datos obtenidos en materia de calidad y seguridad alimentaria. Para ello, se ha dividido la Tesis Doctoral en las siguientes secciones:

Primera sección. Capítulos 1, 2, 3, 4 y 5. Incluye estudios encaminados a modificar el proceso de elaboración de aceitunas de mesa con DOP *Aloreña de Málaga* para reducir el contenido en sodio y mejorar la calidad de los frutos, así como la introducción de nuevas herramientas matemáticas (*functional data analysis*) y técnicas *ómicas* (metagenómica) para el estudio de los procesos.

Segunda sección. Capítulo 6. Incluye el mayor estudio realizado en el sector hasta la fecha para determinar el grado de calidad y seguridad alimentaria del proceso de transformación, de cara a la implantación de medidas encaminadas a APPCC y mejoras del proceso productivo. Fruto de este estudio, se ha procedido a proteger intelectualmente mediante secreto industrial un procedimiento científico para la gestión de la calidad y seguridad alimentaria en las empresas que elaboran aceitunas de mesa y que está siendo explotado por la EBT TAFIQs in FOODS.

La Tesis Doctoral se ha llevado a cabo desde un enfoque multidisciplinar a lo largo de cuatro años de estudio (2013-2017), utilizando técnicas clásicas de microbiología, biología molecular, herramientas ómicas, microbiología predictiva, química analítica y análisis sensorial, a lo que hay que sumar un considerable número de visitas a las empresas para la obtención de muestras para analíticas físico-químicas y microbiológicas y determinación de APPCC. Los resultados que se muestran a continuación son fruto de la realización de una serie de trabajos científicos que se presentan como compendio de publicaciones de acuerdo con la Normativa Reguladora de los Estudios de Doctorado de la Universidad de Córdoba, propuesta por la Comisión de Másteres y Doctorado de 14 de diciembre de 2011 y aprobada por Consejo de Gobierno de 21 de diciembre de 2011.

Finalmente, mencionar la presión que el sector de la aceituna de mesa está sufriendo por parte de los mercados para bajar los niveles de sodio en sus productos, por lo que existe una necesidad en las empresas de bajar los niveles de NaCl que actualmente se utilizan en el proceso de transformación y envasado. Con la actual legislación sobre aceitunas de mesa que existe en España (Real Decreto 679/2016), se da una mayor libertad a las empresas para envasar en cuantos a niveles de pH, sal y acidez libre se refiere con respecto al antiguo reglamento. Sin embargo, en la legislación en vigor se especifica claramente que es responsabilidad de la empresa garantizar la seguridad microbiológica del producto final y aportar estudios y elementos que así lo demuestren frente a las autoridades competentes.

Las principales conclusiones derivadas de esta Tesis, y que se exponen al final del manuscrito, sin duda ayudarán al sector de la *Aloreña de Málaga*, y al de la aceituna de mesa en general, a la elaboración de un producto con una mayor estabilidad, calidad y seguridad microbiológica. Los resultados generados en el presente estudio constituyen una fuente de información relevante para la comunidad científica, el sector industrial y las autoridades sanitarias en materia de calidad y seguridad alimentaria, siendo la base a su vez para el desarrollo de futuros trabajos científicos.

1. INTRODUCCIÓN

1.1. El sector de la aceituna de mesa en cifras

El monocultivo del olivo (*Olea europaea* var. *sativa*) se ha convertido en elemento indispensable del ámbito agrario como consecuencia de una doble conjunción de factores, por un lado, su valía tradicional como proveedor de la principal grasa vegetal en los hogares de la cuenca mediterránea y, por otro lado, su alta rentabilidad desde finales de los 80 con la incorporación de las ayudas europeas y la internacionalización del producto, principalmente aceite y, de forma progresiva, el valor de la aceituna de mesa en sus distintos estilos de elaboración.

Según el Consejo Oleícola Internacional (COI, 2004): "*la aceituna de mesa es el producto preparado a partir de frutos sanos de variedades de olivo cultivado que han alcanzado un grado de maduración apropiado para su procesamiento y que han sido elegidas por producir frutos cuyo volumen, forma, proporción de pulpa respecto al hueso, delicadeza de la pulpa, sabor, firmeza y facilidad para separarse del hueso los hacen particularmente aptos para la elaboración; sometidos a tratamientos para eliminar el amargor natural y conservados mediante fermentación natural o tratamiento térmico, con o sin conservantes, envasados con o sin líquido de gobierno".*

España es el primer país productor de aceitunas de mesa del mundo, seguido muy de cera de otros países como Egipto, Turquía, Argelia y Marruecos (todos ellos de la cuenca mediterránea). Según los datos Consejo Oleícola Internacional (COI, 2017), se estima que existen a nivel mundial unos 850 millones de olivos productivos, plantados en una superficie de 10 millones de Has, de las cuales, no más de 1 millón se dedican a la producción de aceituna de mesa. La producción media mundial de aceitunas de mesa durante las últimas cinco campañas de recogida (2012-2017) ascendió a un total de 2.620.600 ton/año, de las cuales 542.300 se produjeron en España, es decir, un 21% del total (COI, 2017). La superficie de olivar en España se estima en más de 2,5 millones de Has, de los las cuales, cerca del 6% se dedican a la producción de aceituna de mesa y de esta, a su vez, más del 83% están en Andalucía, siendo la principal región productora y exportadora de aceituna de mesa a nivel mundial, principalmente, bajo la transformación de esta drupa carnosa según el "estilo sevillano o español". De este modo, el sector se caracteriza por ser uno de los más

importantes de la industria agroalimentaria española, aportando al PIB de nuestro país aproximadamente 1.100 millones de euros/año (ASEMESA, 2017).

La norma de estándares de calidad comercial del COI (2004) indica que, dependiendo de la forma de eliminar el amargor de las aceitunas se tendrán las siguientes preparaciones comerciales:

·**Aceitunas aderezadas**: *"aceitunas verdes, de color cambiante o negras sometidas a un tratamiento alcalino y acondicionadas en salmuera, donde sufren una fermentación total o parcial, conservadas con o sin acidificantes."*

·**Aceitunas al natural***: "aceitunas verdes, de color cambiante o negras tratadas directamente con una salmuera, donde sufren una fermentación total o parcial, y conservadas con o sin acidificantes."*

·**Aceitunas deshidratadas y/o arrugadas***:* "*aceitunas verdes, de color cambiante o negras, sometidas o no a un ligero tratamiento alcalino, conservadas en salmuera o parcialmente deshidratadas con sal seca y/o aplicando calor o cualquier otro proceso tecnológico*".

·**Aceitunas ennegrecidas por oxidación***:* "*aceitunas verdes o de color cambiante conservadas en salmuera, fermentadas o no, ennegrecidas por oxidación en medio alcalino y conservadas en recipientes herméticos mediante esterilización térmica. Su coloración negra es uniforme*".

1.2. Generalidades de la aceituna de mesa con DOP *Aloreña de Málaga*

La aceituna *Aloreña de Málaga* se enmarcaría dentro de las aceitunas naturales, sin tratamiento con álcali y en concreto: i) por el grado de maduración son "aceitunas verdes", ii) por la preparación comercial se trata de "especialidades", iii) por la presentación: "aceitunas machacadas o partidas", y iv) por los defectos y tolerancias que presenten se clasifican en categoría extra" (suprema) y "categoría primera" (superior).

La *Aloreña de Málaga* es poseedora del distintivo DOP debido a una serie de peculiaridades (elaboración artesana, apreciadas características organolépticas y gran valor nutricional) que han posibilitado una transformación del fruto muy particular en un territorio

concreto, siendo una variedad de olivar exclusiva del área geográfica de la "Olla de Málaga", tierras bajas y falda de las montañas que bordean el cauce del Rio Guadalhorce y sus tributarios. De la importancia de la aceituna "*Aloreña de Málaga*" en la economía local, hablan sus olivares centenarios guardianes de ese frágil equilibrio entre hombre y naturaleza en el entorno de la Sierra de las Nieves y del Valle del Guadalhorce, territorio catalogado como Reserva de la Biosfera en 1995 y con anterioridad Parque Natural de la Sierra de las Nieves. Principalmente, los olivares se asientan en la zona de transición, de ahí la vital importancia de este cultivo en la economía rural de estos territorios del interior tradicionalmente agrícola. El área geográfica que comprende la DOP *Aloreña de Málaga* comprende 19 términos municipales en las comarcas naturales del Valle del Guadalhorce y la Sierra de las Nieves con una superficie de olivar plantado de 17.800 Has y un censo de olivar estimado superior a 1,2 millones de árboles, en su mayor parte, olivar centenario aunque a finales de los 80 se sembraron nuevas plantaciones, anteriormente dedicadas al cultivo de cereal. Por lo tanto, se trata de un cultivo muy localizado altamente influenciado por su ubicación geográfica a la vez que por las técnicas de manejo que junto con el bajo contenido en oleuropeína del fruto, ha derivado en una preparación de las aceitunas para su conservación muy específica y diferente a cualquier otra.

Los últimos datos de los que dispone el Consejo Regulador de la DOP *Aloreña de Málaga* (2016), muestran que el sector tiene una producción media anual de 3.500-4.000 ton, con una estimación de volumen de negocio entre 2-4 millones de euros. A pesar de su aparente escaso volumen de producción, la ratio de generación de mano de obra es muy superior a cualquier otra variedad puesto que su recolección y procesado es 100% manual. Así, se estima que un trabajador es capaz de recolectar entre 150 – 200 Kg/día y la más potente de la agroindustria actual relacionada con la *Aloreña de Málaga* no es capaz de procesar más de 30.000 Kg/día con el empleo de 7 a 9 trabajadores/as. El número de empresas (todas ellas de pequeño tamaño, siendo MicroPymes y Pymes con menos de 20 trabajadores) que elaboran este tipo de especialidad de aceituna de mesa es en la actualidad de 23, dando empleo y rentas complementarias a unas 4.000 familias incluyendo también las actividades relacionadas con la recolección, gestión del olivar e industrias auxiliares. Un producto con un sello de calidad como es la DOP es altamente apreciado en mercados internacionales, y la *Aloreña de Málaga* ya ha sido vendida en países como Alemania, Francia, Holanda, Irlanda,

Italia, Polonia, Reino Unido, Japón y República Dominicana, aunque la mayor parte de sus ventas se realiza en la provincia de Málaga y sobre todo en la Costa del Sol.

Las características que principalmente diferencian a este tipo de variedad de aceituna de mesa y que le han permitido obtener la DOP son las siguientes:

· Son aceitunas curadas, procesadas de manera natural en salmuera.

· Hueso flotante; desprendimiento fácil y limpio del hueso y la pulpa.

· Reducido contenido en grasa y compuestos fenólicos.

· Alto contenido en fibra y conservación de la piel durante el proceso de entamado.

· Características organolépticas claramente diferenciadas de otras aceitunas que empleen la sal como liquido de gobierno para su comercialización.

· Pulpa blanda y sensible a los tratamientos térmicos, aunque crocante con curados solo a base de salmuera.

1.3. Principales tipos de elaboraciones de aceitunas de mesa con DOP *Aloreña de Málaga*

Siglos de tradición arropan/respaldan los diferentes estilos de elaboración de la aceituna *Aloreña de Málaga*, con notables peculiaridades desde su recolección hasta su aderezo y comercialización. Aunque se están modernizando ciertos procesos, en general, la elaboración sigue siendo aún muy artesanal como consecuencia de las peculiaridades del fruto, desde su recolección hasta su clasificado, transformado y envasado. Igualmente, la DOP dispone del oportuno reglamento donde se establece los requisitos y procedimientos de transformación específicos y autorizados para esta aceituna, puesto que se trata de una norma de calidad con la que se pretende dar uniformidad en la transformación del producto comercializado bajo el distintivo DOP *Aloreña de Málaga.*

El inicio de la campaña se define en función del estado de maduración del fruto, aunque esta suele coincidir a mediados o finales de septiembre y se prolonga durante, aproximadamente, 30/40 días hasta mediados o finales de octubre. La recolección se realiza 100% manual debido a lo sensible que es el fruto a los golpes (molestado), empleándose el sistema de ordeño sobre unos recipientes colgados del cuello del agricultor/a llamados "cenachos o macacos". Dado que se trata de una recolección manual, se van seleccionando las aceitunas de mayor calibre y mejor aspecto, sin daño aparente, que se depositan en cajas de plástico destinadas al efecto para evitar apelmazamientos, etc. con un peso aprox. de 20 Kg netos.

En lo que a su elaboración se refiere, el reglamento y pliego de condiciones de la DOP (BOJA nº51, 2015) distingue tres tipos de elaboraciones, dependiendo del grado de fermentación de las aceitunas y características físico-químicas y organolépticas de los frutos:

i) ***Aceitunas Aloreñas Verdes Frescas.*** Una vez las aceitunas han sido recolectadas, se clasifican, lavan y se procede al machacado o partido de los frutos. Posteriormente, se colocan los frutos en bombonas (220L) para su endulzado con una salmuera entre 7 y 11% NaCl. Las bombonas se pueden conservar bajo sombrajos o nave cubierta, o bien en cámaras frigoríficas (máximo 8ºC), donde podrán permanecer mientras no cambien las características que definen esta forma de preparación. La fermentación que sufren los frutos en este proceso es prácticamente nula ya que la conservación en frío ralentiza cualquier cambio, manteniendo los frutos gran parte de las características de la materia prima. Durante esta fase las aceitunas deben mantener el color verde y su textura crujiente. Una vez que se considera que se han endulzado lo suficiente, lo cual se consigue en un mínimo de 3 días, se sacan de las bombonas para continuar la preparación, añadiéndose los aliños típicos que le dan su característico aroma y sabor (ajo, tomillo, hinojo y pimiento) en una proporción que suele ir del 1-3% en el momento del envasado. El pliego de condiciones también permite la utilización de especias u oleorresinas basadas en esos mismos productos naturales. La salmuera madre en envasado se añade a una concentración tal que en el equilibrio esté en torno al 5-6% NaCl. En el envasado está permitida la adición de diferentes conservantes y aditivos entre los que destaca el sorbato potásico, ácido cítrico y benzoato sódico. Para alargar el periodo de comercialización, se puede someter a algún tipo de tratamiento, como puede ser la pasteurización o el envasado al vacío con atmósfera modificada, siempre que el producto final conserve sus características

organolépticas y físicas originarias. Un tratamiento térmico excesivo de los frutos supone una pérdida de calidad del producto, al degradarse su textura, pérdida del apreciado color verde y la aparición de sabor a cocinado (López-López y Garrido-Fernández, 2010), por lo que no es muy habitual su realización.

Las aceitunas verdes frescas se caracterizan por presentar una coloración verde clara, con un olor a fruta verde y a hierba muy agradables que sugieren su frescor y cercana recolección en el tiempo. Asimismo, se nota la presencia de los aliños característicos de su elaboración. Como sabores básicos mencionar que el amargor es la nota característica, así como en ocasiones se puede notar la presencia del salado según las características de su aderezo. La astringencia y el picor son también descriptores que aparecen. Las características físico-químicas que caracterizan a esta forma de preparación de aceitunas son pH comprendido entre 4,2-4,3 y acidez libre entre 0,3-1,0%.

ii) Aceituna Aloreña de Málaga tradicional. En esta preparación las aceitunas se parten y se colocan en bombonas (220L) bajos sombrajos con una salmuera de 11% NaCl por un periodo mínimo de 20 días. Estas aceitunas se comercializan según demanda y no alcanzan las mejores condiciones de calidad hasta bien iniciada la primavera. En este caso se produce una fermentación parcial o total (según el tiempo y temperatura de conservación de las bombonas) por levaduras en una primera etapa y por BAL en una segunda (Arroyo-López, 2007). Posteriormente, se envasan de la misma forma que las aceitunas verdes frescas.

En esta elaboración los frutos presentan una coloración verde-amarillo pajizo, no presentando en esta ocasión un verde intenso. Su olor sugiere a la fruta fresca y a los aliños propios de su aderezo, no percibiéndose las notas a hierba fresca propias de las aceitunas verdes frescas. De su textura, cabe destacar que se trata de una aceituna menos firme, pero que sigue manteniendo sus propiedades en cuanto a lo crujiente, buena separación de la carne con respecto al hueso y a la presencia de piel. De sabor ligeramente amargo es una aceituna menos astringente y de picor menos apreciable que las verdes frescas. Las características físico-químicas que se especifican para esta preparación son un pH que oscila entre 4,0 y 4,3 y una acidez libre entre 0,4 y 1,5%.

iii) Aceituna Aloreña de Málaga curada. En este caso los frutos no se parten antes de la colocación en salmuera, sino que se introducen directamente en grandes fermentadores

(16.000 L de volumen) empleándose una salmuera con una concentración que varía entre el 6,0 y 7,5% NaCl junto con una concentración de ácido acético que puede variar entre el 0,5% y el 0,8%. En esta forma de preparación, se suele añadir el ácido acético con el fin de facilitar la fermentación natural de las aceitunas y asegurar su buena conservación. Las aceitunas pueden conservarse en estas condiciones durante periodos prolongados de tiempo, siendo el tiempo mínimo de permanencia en fermentadores de 90 días, lo cual garantiza una fermentación total del proceso, llevado a cabo fundamentalmente por levaduras y BAL (Arroyo-López, 2007). Posteriormente, se produce el machacado, aliñado y envasado de los frutos como en las elaboraciones anteriores.

La aceituna curada se caracteriza por presentar una coloración amarilla-marrón, con un olor a fruta madura y a hierba fresca. Se nota la presencia de los aliños y de notas lácticas, características de su elaboración y del proceso de fermentación. De textura menos firme y crujiente, presentan una buena separación de la carne con respecto al hueso, así como se manifiestan restos de la presencia de piel tras su masticación. De sabor ácido, pierden su amargor resultando picantes tras su degustación. Las condiciones físico-químicas que caracterizan este tipo de producto oscila en un pH entre 3,3 y 3,8 y una acidez libre entre 1,5 y 3,0%.

1.4. Necesidades del sector

El efecto de la globalización por la entrada en el mercado de nuevos productores más competitivos ha provocado el desarrollo de nuevos métodos de cultivo súper intensivo que están asociados a una estructura de la propiedad de la tierra claramente definida (Malefakis, 2001). Ello propicia la exacerbación de los monocultivos industriales que están y continuarán desplazando a otras variedades con menos posibilidades tecnológicas dentro de la agroindustria (como es la aceituna *Aloreña de Málaga),* todo ello derivado de la reducción de los objetivos empresariales a uno solo, la rentabilidad a cualquier coste ya sea humano o medio ambiental. Por lo tanto, la distorsión del concepto innovación que tiene como consecuencia la supresión de variedades minoritarias hacia monocultivos con una reducida variabilidad genética puede potencialmente encaminar al sector del olivar a una debilidad manifiesta ante la globalización biológica, donde la movilidad de patógenos como está siendo el caso de *Xylella fastidiosa* puede tener efectos económicos y empresariales equivalentes a

un deficiente diseño de la estrategia industrial de la empresa. De este modo, la preservación de cultivos y estilos de transformación minoritarios apoyados con distintivos de calidad diferenciadores que, no solo resalten las cualidades saludables del producto, sino las bondades de su cultivo en lo que a impacto ambiental y social provocan en los territorios a la vez que evitan la despoblación de estos y genera una economía más sostenible y sustentable, debe ser impulsada y apoyada directa e indirectamente por las administraciones públicas. Es en este punto donde los centros de investigación y la cooperación con el sector se visualiza y se pone en práctica en trabajos como la presente Tesis Doctoral, donde se consigue dar un impulso a la innovación y modernización de procesos tecnológicos e instrumentos de gestión que, de otra forma serían difíciles o, directamente, inalcanzables para el ecosistema micro-agro-industrial andaluz.

Actualmente, en España el sector de la aceituna de mesa tiene que resolver diferentes retos entre los que destacan:

· La problemática nutricional derivada de su alto contenido en sodio y grasa.

· Baja penetración en mercados emergentes como el asiático.

· La bajada de su ingesta per cápita al ser desplazado por otros aperitivos de nueva aparición.

· Tratamientos y gestión de vertidos.

· Presión por terceros países en los que los costes de producción son muchos menores.

En el caso específico de la aceituna *Aloreña de Málaga*, se han detectado las siguientes problemáticas que deberían y podrían ser corregidas:

· Escasos recursos hídricos que aumentan el problema de la vecería, agravada al no realizarse adecuadas prácticas de cultivo y explotación de los acuíferos.

· Falta de personal técnico cualificado en la cadena productiva, desde el cultivo hasta la comercialización del producto.

· Poca estandarización en el proceso de producción, lo cual repercute directamente en un producto de menor calidad y poco homogéneo.

· Grandes diferencias tecnológicas en los diferentes pasos integrantes del proceso productivo.

· Bajo nivel productivo, tanto de explotaciones agrícolas como de la agroindustria, todo ello derivado de una deficiente gestión de procesos e implementación de tecnología, la máxima que la elaboración permita sin comprometer el valor diferencial del producto final.

· Falta de transferencia de conocimientos a los actores de la cadena de producción y comercialización.

· Bajos márgenes de comercialización y, por tanto, notable reducción de los porcentajes de beneficios en los últimos años lo cual se ve agravado por la dimensión de la agroindustrial que, como se ha hecho referencia anteriormente, son pequeñas con lo que tienen poco margen para provocar una alteración en los umbrales de rentabilidad.

· Alta inestabilidad de los envasados, que limita las ventas a lugares cercanos a su zona de producción y reduce sus posibilidades de acceder a nuevos mercados y/o canales de distribución.

Las razones por las cuales no existe una comercialización generalizada y globalizada de aceitunas *Aloreñas de Málaga*, sostenida en el tiempo y capaz de generar una demanda creciente que empuje al agricultor a incrementar la producción y/o estabilizar la existente mediante la modernización de las explotaciones tiene su origen en las dificultadas de conservación para que lleguen a los canales de distribución con el tiempo suficiente requerido, de ahí la importancia del presente trabajo como impulsor de un cambio de tendencia en lo que a la transformación se refiere a la vez que la inclusión de la innovación y el desarrollo en el ADN de las empresas aderezadoras, aunque sea de forma agregada entre agrupaciones de ellas.

Cada vez más, los consumidores demandan alimentos con mayor calidad, mayores cotas de seguridad y con mayor valor funcional. En este sentido, la reducción del contenido en sodio en el proceso de elaboración de la aceituna se ha convertido en una prioridad para el

sector (Bautista-Gallego et al., 2013). En el caso de la aceituna *Aloreña de Málaga* que se trata de una aceituna partida, en el que la entrada de sal al fruto se lleva a cabo de una manera más rápida, lo cual provoca que, en este tipo de elaboración se aprecie aún más el sabor salado. Por tanto, las empresas que elaboran aceitunas *Aloreñas de Málaga* llevan años interesadas en reducir el uso de NaCl, tanto durante el proceso de elaboración como en el envasado (Bautista-Gallego, 2012). En este sentido, la búsqueda de otras alternativas al proceso de elaboración como es la conservación de aceitunas en un medio ácido (Garrido-Fernández y col., 1997), podría reducir el uso de sales, lo cual también supondría un efecto colateral positivo, tanto así como una mejora en el tratamiento de los vertidos, lo cual se está convirtiendo en un grave problema medioambiental derivado de la falta de desarrollo tecnológico adecuado para la valorización de los efluentes con alta conductividad.

Otra demanda del sector es la conservación del color verde de los frutos durante el mayor tiempo posible, así como la reducción /reversión del molestado de las aceitunas debido a los golpes que los frutos se llevan durante el proceso de recolección. Un tratamiento térmico de las aceitunas, justo después de su recolección, qué inactiven ciertas enzimas que dan lugar a los procesos de pardeamiento podrían tener también aplicación en el proceso de elaboración de aceitunas *Aloreña de Málaga* (Ramírez et al., 2015a).

La aceituna *Aloreña de Málaga* es un producto que, en los últimos años, ha recibido una gran atención por parte de la comunidad científica debido a sus singularidades y su propensión a ser empleada como modelo de estudio. En este sentido, una búsqueda en PUBMED con la palabra clave *Aloreña* refleja que desde el 2005 se han realizado un total de 26 publicaciones científicas, 3 tesis doctorales (Arroyo-López, 2007; Casado-Muñoz, 2016; Romero-Gil, 2018) y se ha editado 1 libro (López-López y Garrido-Fernández, 2010), todos ellos encaminados a obtener un mayor conocimiento de los procesos, la búsqueda de microorganismos con potencial probiótico y la mejora del proceso de elaboración. Sin embargo, en la actualidad, existen nuevas herramientas científicas como la metagenómica y el empleo de diferentes modelos predictivos, que podrían aplicarse a la aceituna *Aloreña de Málaga* para un mejor conocimiento de los procesos de elaboración e identificar potenciales peligros y alterantes, con la intención de mejorar la gestión de la calidad y seguridad alimentaria.

1.5. Aplicación de nuevas herramientas y procedimientos científicos al proceso de transformación de la aceituna de mesa con DOP *Aloreña de Málaga*

1.5.1. Microbiología predictiva

Los sistemas alimentarios conforman estructuras generalmente complejas donde los microorganismos pueden presentar un comportamiento variable en función de las condiciones de proceso y factores ambientales presentes. En muchos alimentos, entre ellos, los vegetales fermentados, coexisten diferentes poblaciones microbianas que presentan fenómenos de sinergismo, antagonismo o comensalismo entre ellas. Esto hace que el alimento sufra una serie de modificaciones que en ocasiones son difícilmente predecibles. Por ello, se hace necesario disponer de herramientas capaces de predecir el comportamiento de los microorganismos en alimentos en condiciones previsibles de almacenamiento y procesado. Para ello, se requiere un exhaustivo control de los principales factores físico-químicos que pueden ejercer una influencia (temperatura, pH, NaCl etc.) así como un conocimiento profundo acerca del comportamiento microbiano frente a dichos factores. Si a esto le unimos las actuales técnicas y metodologías para la gestión masiva de datos, lo que se ha venido a llamar "Big-Data", las posibilidades de desarrollar herramientas que faciliten la labor y maximicen la seguridad alimentaria del producto, son elevadas, tanto es así que, como fruto de la presente Tesis Doctoral se ha desarrollado una propuesta (software) que canaliza dicha información y reporta datos elaborados que ayudan a la toma de decisiones que revierten directamente en la seguridad del producto final.

Los análisis microbiológicos requieren de tiempo, material y recursos que, generalmente, son elevados y no siempre disponibles de forma inmediata. Además, los resultados generados llevan aparejados una variabilidad asociada a las cepas microbianas, condiciones de cultivo y matriz del alimento objeto de estudio.

La microbiología predictiva es una rama especializada de la microbiología de los alimentos dedicada a estudiar y predecir el comportamiento microbiano frente a factores ambientales e intrínsecos al microorganismo, haciendo usos para tal fin, de funciones matemáticas (McMeekin et al., 1993).

La microbiología predictiva constituye una rama dentro de la microbiología de alimentos que trata de evaluar de forma cuantitativa las respuestas asociadas al comportamiento de los microorganismos en alimentos mediante el desarrollo y aplicación de modelos de predicción. Dichos modelos comportan ecuaciones matemáticas capaces de reflejar los principales factores relacionados con el crecimiento, supervivencia o inactivación de los microorganismos frente a distintas condiciones de procesado y almacenamiento. Los modelos de predicción constituyen por tanto una valiosa herramienta utilizada en el ámbito industrial y gubernamental. En el sector de la aceituna de mesa, algunas de las posibles aplicaciones se recogen en la Tabla 1. Es necesario tener en cuenta que los modelos constituyen simplificaciones de un sistema complejo por lo que los resultados proporcionados por un modelo de predicción deben ser validados en el alimento de interés antes de su aplicación.

Tabla 1. Posibles aplicaciones de la microbiología predictiva en el sector de la aceituna de mesa

Análisis de Peligros y Puntos de Control Crítico (APPCC)
✓ Análisis preliminar de peligros en la recepción de materias primas ✓ Identificación y establecimiento de puntos críticos de control ✓ Acciones correctoras ✓ Evaluación de la interacción entre variables de proceso
Evaluación de Riesgo Microbiológico
✓ Estimación de los posibles cambios en los niveles de un determinado microorganismo durante el procesado, envasado y almacenamiento. ✓ Evaluación de la Exposición hacia un microorganismo patógeno
Estudios de vida de mercado
✓ Predicción del crecimiento de posibles microorganismos patógenos y alterantes en alimentos
Investigación y desarrollo de nuevos productos
✓ Evaluación del efecto de la alteración microbiana en productos envasados ✓ Efecto de los tratamientos de pasteurización de aceitunas sobre la reducción de la contaminación microbiana ✓ Evaluación del efecto de circunstancias externas al control de producción
Efecto de formulaciones alternativas
✓ Evaluación de la adición de conservantes sobre la calidad y seguridad del producto final
Educación
✓ Educación de personal científico y no científico
Diseño de experimentos
✓ Número de muestras a preparar ✓ Definición de los intervalos entre cada factor a analizar

El desarrollo de modelos de predicción se inició en 1921 cuando Bigelow describió la relación entre el logaritmo neperiano de la tasa de inactivación y la temperatura para definir la destrucción de esporas de *Clostridium botulinum* en alimentos enlatados. Sin embargo, no fue hasta 1980 cuando comenzó el mayor desarrollo de la microbiología predictiva. La premisa en la que se basan la mayoría de los modelos es que el comportamiento microbiano puede ser reproducible frente a diversos factores extrínsecos e intrínsecos (Ross et al., 2000). Este comportamiento se traslada a diversas funciones matemáticas que definen el crecimiento/inactivación/producción de toxina/ probabilidad de crecimiento etc. Esta concepción se conoce como el modelado de las respuestas microbianas en alimentos y aparece en diversas publicaciones bibliográficas (McKellar & Lu, 2004; Brul et al., 2007; Pérez-Rodríguez y Valero, 2013; Arroyo-López et al., 2014).

Distintos autores señalan varias clasificaciones de los modelos de predicción. La más comúnmente usada es la propuesta por Whiting y Buchanan (1994) en función de su grado de desarrollo:

· **Modelos primarios:** Se definen como funciones matemáticas que describen la cinética de los microorganismos en función del tiempo. La mayor parte de las funciones (ej. Baranyi, Gompertz, modelo logístico etc.) son de tipo sigmoide y tienen por objeto cuantificar los parámetros cinéticos asociados al crecimiento e inactivación microbianas:

- Tasa máxima de crecimiento (μ_{max}, log ufc/g) definida como el incremento en el número de unidades logarítmicas en función del tiempo. Dicho parámetro describe la velocidad de crecimiento microbiano y está asociado al tiempo de duplicación (TD, h) definido como el tiempo necesario para que la población microbiana (cfu/g) se multiplique por dos. El parámetro μ_{max} también puede estar definido en unidades de logaritmo neperiano, en cuyo caso se denomina tasa máxima específica de crecimiento (h^{-1}).
- Fase de latencia (λ, h) que describe el tiempo de adaptación del microorganismo al medio o alimento. El parámetro λ es dependiente tanto de las condiciones ambientales de crecimiento como las condiciones fisiológicas del microorganismo previas a su colonización en el medio.

- Densidad máxima de población (N_{max}, log UFC/g) que se define como el tamaño máximo de población que puede alcanzarse bajo unas condiciones ambientales definidas.
- Tasa máxima de inactivación (k_{max}, log UFC/h): que describe el descenso en el número de unidades logarítmicas frente al tiempo. Representa la capacidad que posee un tratamiento de inactivación de destruir la población microbiana en un tiempo determinado. El parámetro k_{max} se relaciona de forma inversamente proporcional con el valor D (tiempo de reducción decimal).

· **Modelos secundarios:** Los modelos secundarios son funciones matemáticas que relacionan las condiciones ambientales o de proceso estudiadas frente a los parámetros cinéticos estimados por los modelos primarios. Los modelos secundarios se clasifican en distintas tipologías siendo las más representativas las siguientes:

- Modelos polinómicos: son funciones cuadráticas que presentan un alto número de parámetros y por tanto describen de forma empírica el comportamiento microbiano. Se caracterizan por ser funciones flexibles capaces de modelar la interacción de los factores ambientales con suficiente precisión.
- Modelos raíz cuadrada o de Ratkowsky (Ratkoswky et al., 1995) relacionan la raíz cuadrada de μ_{max} con los factores ambientales.
- Modelo Gamma: describe el efecto multiplicativo de los factores ambientales sobre μmax basado en el cálculo de los valores mínimos, máximos y óptimos de los factores ambientales (Zwietering et al., 1996).

· **Modelos terciarios:** Los modelos terciarios integran la información de los primarios y secundarios en plataformas informáticas de fácil manejo para que los modelos puedan ser aplicados por los usuarios a nivel industrial o para desarrollar procesos de toma de decisiones. Algunos de los softwares más representativos son:

- Combase (Institute of Food Research, Norwich, Reino Unido, www.combase.cc);
- Pathogen Modeling Program v8.0 (United States Department of Agriculture, Estados Unidos, https://www.ars.usda.gov/northeast-area/wyndmoor-pa/eastern-regional-research-center/residue-chemistry-and-predictive-microbiology-research/docs/pathogen-modeling-program/pathogen-modeling-program-models/);

- Food Spoilage and Safety Predictor v4.0 (Technical University of Denmark; http://fssp.food.dtu.dk/);
- MicroHibro v2.0 (Universidad de Córdoba; www.microhibro.com).

1.5.2. Estudios de ecología microbiana aplicados en aceitunas de mesa

La fermentación de aceitunas de mesa es un proceso dinámico y complejo en el que la microbiota sufre cambios secuenciales a lo largo del proceso de elaboración. Existen diversos estudios sobre la microbiota en las aceitunas de mesa dependiendo del tipo de cultivo, país de origen, métodos de elaboración, etc., con el objetivo de controlar los procesos fermentativos, evitar alteraciones no deseadas, seleccionar cultivos iniciadores adecuados para optimizar el proceso de elaboración y mantener la calidad y seguridad del producto final.

La mayoría de los estudios para describir la microbiota en las aceitunas de mesa han sido llevados a cabo mediante técnicas de microbiología clásica dependientes de cultivo, siendo necesario el aislamiento previo de los microorganismos predominantes desde la matriz del alimento para así realizar la posterior caracterización por análisis bioquímicos o moleculares. Algunos estudios en aceitunas de mesa consiguieron una diferenciación inicial mediante una caracterización bioquímica de los aislamientos permitiendo discriminar cepas con los mismos fenotipos, pero con diferentes características metabólicas, como por ejemplo las basadas en los distintos patrones de fermentación de azúcares (Randazo et al., 2004). No obstante, esta técnica no logró conseguir la caracterización de un gran número de especies y géneros.

En la actualidad, los métodos moleculares son los más empleados para la identificación y caracterización de los microorganismos asociados a alimentos. La amplificación parcial por PCR del ADN o ARN extraído directamente de colonias aisladas de los microorganismos de interés, y su posterior secuenciación, permite obtener una identificación con mucha mejor precisión gracias a la existencia de las bases de datos, como GenBank (Altchusl et al., 1990). La amplificación parcial por PCR del gen que codifican al rRNA 16S ha sido una herramienta muy útil para identificar y clasificar a un gran número de bacterias (Weisburg et al., 1991). Asimismo, la secuenciación parcial del gen 28S rRNA y/o de la región ITS del ADN de levaduras y mohos han sido lo más extendidos (Esteve- Zarzoso et al., 1999; Kurtzman and Robnett, 1998).

A diferencia de las reacciones de PCR tradicionales, otras técnicas moleculares utilizan el ADN polimórfico amplificado al azar (RAPD) o los elementos repetitivos del ADN bacteriano (rep-PCR) mediante el uso de cebadores cortos (Williams et al., 1990; Gevers et al., 2001). Ambas técnicas, crean fragmentos con diferentes longitudes originando un perfil de bandas específico para cada cepa. Una caracterización inicial a través de REP o RAPD permite agrupar en clusters diferentes microorganismos con perfiles similares, y mediante la posterior secuenciación, se puede confirmar la identificación de los mismos. En los últimos años, muchos estudios han utilizado este enfoque molecular en el campo de las aceitunas de mesa (Hurtado et al., 2008; Aponte et al., 2010; Bautista-Gallego et al., 2011; Franzetti et al., 2011; Tofalo et al., 2013; Lucena-Padrós et al., 2014). Estas herramientas moleculares son muy populares para caracterizar y realizar estudios filogenéticos de comunidades microbianas. En particular, en la aceituna *Aloreña de Málaga*, Abriouel et al. (2012) realizó una identificación de la población de BAL asociadas a este tipo de elaboración de aceitunas de mesa mediante la secuenciación de los genes *phes* y *rpo*. Asimismo, Romero-Gil et al. (2016) estudiaron las poblaciones de *Enterobacteriaceae y Lactobacillaceae* mediante la secuenciación del gen 16S del rDNA y los genes *recA* respectivamente.

Las técnicas moleculares son rápidas, fáciles de realizar, más precisas y eliminan la subjetividad que generalmente ocasionan las pruebas bioquímicas. Sin embargo, los métodos dependientes del cultivo presentan dos grandes inconvenientes: i) el aislamiento lleva días o incluso semanas, y ii) solo se detectan microorganismos viables. Las técnicas dependientes de cultivo no ofrecen un perfil completo de la diversidad microbiana presente en un ecosistema específico o matriz alimentaria ya que solo pueden detectar a las células viables y cultivables con esta metodología. Por esta razón, los métodos independientes de cultivo han atraído la atención de muchos científicos en la rama de la ciencia de los alimentos.

Entre los métodos independientes de cultivo la PCR junto con la electroforesis en gel con gradiente desnaturalizante (PCR-DGGE) ha recibido una gran atención en el estudio de comunidades bacterianas. La PCR-DGGE separa los productos de PCR a lo largo de un gradiente discriminando por diferencias en las secuencias. Recientemente, numerosos estudios de ecología microbiana se han llevado a cabo en aceitunas de mesa utilizando esta técnica (Abriouel et al., 2011; Mucilli et al., 2011; Tofalo et al., 2013; Lucena-Padrós et al., 2015). Estos estudios se han llevado a cabo en las salmueras de fermentación exclusivamente,

sin tener en cuenta la microbiota adherida a la superficie del fruto, que es lo que realmente es ingerido por los consumidores. A pesar de su gran popularidad, la técnica de PCR-DGGE consume mucho tiempo y los gradientes y geles son difíciles de configurar y ejecutar correctamente. Además, no es una técnica cuantitativa, por lo que puede que solamente detecte la presencia de las especies más abundantes, ya que la PCR puede introducir sesgos.

Las nuevas técnicas de secuenciación masiva (NGS), como técnicas independientes de cultivo, incluye varias tecnologías que logran una secuenciación masiva y paralela de segmentos cortos usando cebadores de PCR universales, principalmente para amplificar el 16S rRNA en bacterias y las regiones ITS para levaduras y hongos. Esta nueva tecnología posee muchas ventajas en comparación con los métodos de generación de perfiles de primera generación. Todos los sistemas NGS secuencian de miles a miles de millones de lecturas en una sola ejecución y es más sensible a los OTU (Unidad Taxonómica Operacional) de baja abundancia, siendo más robusto en el estudio de la diversidad. El uso de NGS, incluido dentro de las técnicas ómicas, ha permitido el estudio cuantitativo de las comunidades microbianas en alimentos aportando mayor información de los procesos fermentativos y de la microbiota presente en las materias primas (Ercolini, 2013; Kergourlay et al., 2015).

Estas novedosas técnicas han revolucionado el campo de la ecología microbiana en alimentos a través de una identificación más precisa de taxones microbianos sin la necesidad de métodos dependientes del cultivo. En el caso particular de la aceituna de mesa, Cocolin et al. (2013) y De Angelis et al., (2015) han empleado esta metodología para el estudio de la biodiversidad bacteriana adherida a la superficie de diversas variedades de aceitunas italianas (Nocellare etnea y Bella di Cerignola) utilizando el gen que codifica al rARN 16S. De esta manera, la metagenómica se convierte en una herramienta ideal para el estudio de las poblaciones microbianas en alimentos fermentados, en particular en la aceituna de mesa.

La metagenómica aporta conocimientos sobre los cambios poblaciones, tanto en bacterias como levaduras, desde las materias primas, a lo largo del proceso de elaboración y hasta producto final. Asimismo, permite observar el impacto que sufren los microorganismos ante diversos tratamientos, condiciones de elaboración, influencia geográfica, gestión de la higiene y limpieza en las industrias, origen de las materias primas, etc. Todo ello permitirá obtener una información valiosa del proceso de fermentación y de la comunidad microbiana

para el diseño de nuevas estrategias en aras de mejorar la calidad y la seguridad de la aceituna de mesa.

1.5.3. Tratamientos térmicos aplicados en aceitunas de mesa

En los últimos años, se ha incrementado la demanda por productos más tradicionales, naturales y ecológicos. En el caso de la aceituna de mesa, las elaboradas como verdes naturales o en salmuera son un claro ejemplo de este tipo de productos, a diferencia de las elaboradas como estilo español que hace uso de soluciones de hidróxido sódico. Se pueden encontrar dos grandes diferencias entre ambas elaboraciones de aceitunas tipo verdes teniendo en cuenta los factores indicados anteriormente: i) las aceitunas elaboradas como verdes estilo español presentan un color verde más apreciado por los consumidores que las aceitunas elaboradas como verdes naturales, que presentan un color más pardo; ii) en los frutos elaborados como verdes estilo español se produce una fermentación láctica, que puede no desarrollarse en aceitunas verdes naturales debido a la presencia de compuesto antimicrobianos. Ambos parámetros son de vital importancia para la optimización de nuevos procesos de elaboración. Mantener una coloración verde óptima y permitir una fermentación láctica de las aceitunas son un reto muy perseguido por el sector de la aceituna de mesa.

En la elaboración de aceitunas de mesa tipo verdes, los frutos son recolectados del árbol con una coloración verde amarillenta y pueden ser destinadas a la producción de aceitunas verdes estilo español, cuando se realiza un tratamiento con NaOH, o dar lugar a aceitunas verdes naturales cuando se colocan directamente en salmuera. En ambas elaboraciones el fruto cambia su coloración, presentando un color distinto en el producto elaborado. El color verde de los frutos es debido a la presencia de compuestos clorofílicos, mientras que el color amarillo viene dado por los compuestos carotenoides (Mínguez-Mosquera & Garrido- Fernández, 1989). Sin embargo, la diferencia de color entre ambos tipos de elaboraciones no se debe a las diferencias que estos pigmentos puedan sufrir a lo largo del proceso de transformación de los frutos, sino a procesos de oxidación de polifenoles por la actividad enzimática polifenol oxidasa (PPO) (Segovia-Bravo et al., 2009). Recientemente, Ramírez et al., (2015b) detectó mayores concentraciones de pigmentos verdes al final de la conservación en aceitunas Hojiblanca y Manzanilla elaboradas como verdes naturales, que son las que más se oscurecen, que en aceitunas elaboradas como verdes estilo

español y comprobó que este oscurecimiento era debido a la oxidación enzimática de los compuestos o-difenólicos presentes en los frutos frescos, particularmente en la piel, por la acción de la enzima PPO. En aceitunas verdes elaboradas como estilo español, el tratamiento con hidróxido sódico inactiva a la enzima PPO sin producirse la oxidación enzimática de los compuestos o-difenólicos en los frutos, permitiendo que los frutos mantengan una coloración más verde (Ramírez et al., 2015b).

Además, el tratamiento con álcali en aceitunas elaboradas como verdes estilo español permite un endulzamiento rápido de los frutos en un corto período de tiempo favoreciendo el desarrollo de BAL que permite una fermentación típica y genera un producto con un color, flavor y aroma característico. En cambio, el endulzamiento en aceitunas elaboradas como verdes naturales es un proceso que requiere más tiempo, condicionado por la hidrólisis lenta en condiciones ácidas de la oleuropeína que difunde desde el fruto a la salmuera (Medina et al., 2008). La colocación de las aceitunas en la salmuera provoca la rotura de tejidos en el fruto facilitando el contacto de los compuestos fenólicos y las enzimas endógenas de los frutos. De esta manera, la oleuropeína puede ser sustrato de la enzima β-glucosidasa y originar la forma dialdehídica decarboximetilada del ácido elenólico unido a hidroxitirosol (HyEDA). Este compuesto tiene una reconocida actividad antimicrobiana, similar a la del desinfectante comercial glutaraldehído, y produce la inhibición de las BAL propias de la fermentación de aceitunas de mesa (Medina et al., 2007).

Desde los años 70 se conoce que el tratamiento térmico de las aceitunas Manzanilla favorece el crecimiento posterior de los lactobacilos en la salmuera, aunque no se llegó a dar ninguna explicación a este fenómeno (Fleming et al., 1973; Rodríguez-Borbolla et al., 1979). Balatsouras et al. (1983) también encontró una ligera mejora en la fermentación de aceitunas verdes de la variedad *Conservolea* mediante la aplicación de un tratamiento térmico. Posteriormente, Montedoro et al. (2002) observó que la concentración de HyEDA en pulpa de aceitunas era inferior en aceitunas escaldadas que en frutos no calentados. Recientemente, Ramírez et al. (2017) diseñó un nuevo proceso de elaboración de aceitunas verdes naturales mediante tratamientos térmico de los frutos frescos antes de su colocación en salmuera, y concluyó que la aplicación de tratamientos térmicos no demasiado intensos (temperaturas de 60ºC durante 10 minutos) a frutos de aceitunas Manzanilla y Hojiblanca, eran suficiente para inactivar a la enzima β-glucosidasa y, por tanto, evitar la formación de los compuestos

antimicrobianos, favoreciendo así el crecimiento de BAL. Además, las aceitunas tratadas térmicamente presentaban una coloración similar a las elaboradas al estilo español tratadas con NaOH, con un color verde más claro que las no tratadas con calor, debido a la inactivación de la enzima PPO.

A pesar de las ventajas que presenta el calentamiento de los frutos, el inconveniente de someter las aceitunas a tratamientos térmicos elevados puede producir alteraciones en algunos parámetros tales como la textura (Brenes et al., 1994) resultando un producto final con baja aceptación para el consumidor. Ramírez et al. (2017) concluyó que los tratamientos de los frutos por debajo de 80ºC no repercutían en una pérdida importante de textura con respecto al fruto no tratado térmicamente. Además, esta pérdida de textura podía llegar a ser positiva en algunas variedades más duras y fibrosas, como es el caso de la variedad Hojiblanca. Sin embargo, Ramírez et al. (2017) observó que los tratamientos con calor provocaban una mayor liberación de oleuropeína en frutos los frutos de aceitunas respecto a las no tratadas, acentuando el sabor amargo que origina este compuesto, incluso después de 6 meses de fermentación.

Algunas cepas de BAL, entre ellas *Lactobacillus plantarum*, poseen actividad β-glucosidasa y esterasa en su metabolismo y muchos investigadores han estudiado la posibilidad de usar esta capacidad para la hidrólisis de la molécula de oleuropeína en compuestos no amargos y así acelerar el proceso de endulzamiento (Ciafardini et al., 1994; Marsilio et al., 1996; Ghabbour et al., 2011; Zago et al., 2013; Tofalo et al., 2014; Ramírez et al., 2017). Además, estas trasformaciones enzimáticas también fueron estudiadas in vitro con diferentes cepas de levaduras en aceitunas verdes naturales (Bautista-Gallego et al., 2011; Tofalo et al., 2013) y aceitunas negras (Bonatsou et al., 2015) siendo *Wickerhanomyces anomalus* la que presentó mayor actividad. Sin embargo, los estudios a escala piloto, en los que se conseguía un endulzamiento de los frutos, han sido bastantes escasos y ninguno de estos cultivos iniciadores se han aplicado a escala industrial (Servilli et al., 2006; Kaltsa et al., 2015). Ramírez et al. (2017) seleccionó un *Lactobacillus pentosus* con alta capacidad de hidrolizar oleuropeína en soluciones modelos y lo utilizó como inóculo en aceitunas Manzanilla, Hojiblanca, Gordal y *Aloreña de Málaga* tratadas térmicamente. Después de 6 meses de fermentación, los panelistas no encontraron amargor para las aceitunas Gordales y *Aloreña de Málaga* tratadas térmicamente, y al contrario para Manzanilla y Hojiblanca. Este

dato está en consonancia con los encontrados por Medina et al. (2009), ya que las variedades *Aloreña de Málaga* y Gordal poseen una menor concentración de oleuropeína en el fruto fresco. El tratamiento de calor aplicado, a pesar de ser poco agresivo (60 ºC durante 15 minutos) no provoco cambios en la textura de las variedades Manzanilla y Hojiblanca, en cambio originó una pérdida para las variedades Gordal y *Aloreña de Málaga* que la hicieron inaceptable por los consumidores (Ramírez et al., 2017).

En el caso particular de la elaboración de aceitunas *Aloreña de Málaga* como aceitunas verdes en salmuera, el color verde de los frutos es un atributo muy determinante y tenido en cuenta por los consumidores. De esta manera, en las elaboraciones tipo frescas y tradicionales, se pretende obtener un producto con un color verde intenso, característico de este producto. En cambio, en la elaboración tipo curadas, donde el tiempo de conservación en salmuera es mayor, el color se ve más afectado con unas tonalidades más pardas. Con objeto de mitigar el oscurecimiento del fruto se han investigado la aplicación de lavados y atmósferas protectoras de dióxido de carbono (Arroyo-López et al., 2007), el uso de compuestos antioxidantes como ácido ascórbico, metabisulfito de sodio o varias sales minerales de magnesio y zinc (Arroyo-López et al., 2008; Gallardo-Guerrero et al., 2013), pero ninguna de ellas ha sido completamente satisfactoria. Por lo tanto, la aplicación de tratamientos de calor a los frutos de aceitunas *Aloreña de Málaga*, antes de su colocación en salmuera, evidencia una serie de ventajas siendo una alterativa más natural para mejorar la coloración del fruto en el producto final.

A pesar de que la variedad *Aloreña de Málaga* posee una baja o moderada concentración de oleuropeína en el fruto fresco (Medina et al., 2009), al permanecer poco tiempo en salmuera, el producto final posee cierto sabor amargo, más acentuado en la elaboración tipo frescas y menos en las tipo tradicionales y curadas. El sabor amargo característico de las aceitunas *Aloreña de Málaga* es otra cualidad muy apreciada por los consumidores. Por lo tanto, el aumento en la concentración de oleuropeína en las aceitunas *Aloreña de Málaga* tratadas térmicamente no debería suponer un problema, ya que, según estudios previos (Ramírez et al., 2017), el amargor del fruto no resulta desagradable por los consumidores después de la fermentación.

Además, la aplicación de los tratamientos térmicos a los frutos permitió una fermentación láctica de todas las variedades estudiadas (Ramírez et al., 2017). La fermentación láctica de los alimentos, y en particular de la aceituna de mesa, presenta una serie de beneficios con respecto a los no fermentados, entre ellos, mejora las características organolépticas del producto y se consigue aumentar la seguridad microbiológica del producto final extendiendo su vida de mercado. El ácido láctico producido en la fermentación de aceitunas por las BAL, a partir de los azúcares de fruto, otorga acidez al producto final y posee excelentes propiedades conservantes, alcanzando unos valores de pH más bajos que origina un producto final microbiológicamente más estable y seguro.

Con estos antecedentes, los tratamientos térmicos de los frutos de aceitunas presentan grandes ventajas en el desarrollo de un nuevo proceso de elaboración de aceitunas verdes en salmuera. En el caso particular de la aceituna *Aloreña de Málaga*, aún se debe realizar un estudio más en profundidad para optimizar el proceso de elaboración con el fin de obtener un producto final con unas características organolépticas (color, aroma, sabor y textura) apropiadas, y una mayor estabilidad microbiológica del producto, aspectos positivos a considerar para una mayor aceptabilidad del consumidor.

1.6. Análisis de peligros y puntos de control críticos en la industria de la aceituna de mesa

Los procesos llevados a cabo en las industrias alimentarias son generalmente diversos y cada vez más sofisticados como consecuencia de la evolución en la demanda de los consumidores a la vez que una generalización de la necesidad de innovar en dichos procesos para hacerlos más eficientes, eficaces, seguros y replicables. Dichos procesos de transformación tienen como principales objetivos la adecuación y mejora de las condiciones de las materias primas o productos semielaborados de cara a proporcionar alimentos saludables y apetecibles a una población consumidora.

Los cambios en las tendencias de consumo, la democratización y el mayor acceso a la información y formación por parte de los consumidores ha empujado a la agroindustria hacia una obligada modernización de los sistemas de producción de alimentos, lo cual ha traído consigo la necesidad de mejorar los procedimientos, tanto internos (en la fábrica) como externos, tanto en la recolección de los frutos como en la distribución del producto elaborado,

lo que ha llevado inexorablemente a la mejora de la calidad y seguridad alimentaria de los mismos.

La armonización de los sistemas de producción requiere del desarrollo e implantación de herramientas destinadas a controlar los procesos industriales y evitar que se produzcan desviaciones que impliquen la generación de un producto "no seguro" y, por tanto, una posible retirada del mercado de los alimentos finales, con la generación de la consiguiente alarma y/o alerta alimentaria. Para ello, los Sistemas de Gestión de la Seguridad Alimentaria (SGSA) establecen una serie de pautas normativas mediante las cuales los industriales deben garantizar que los alimentos producidos puedan comercializarse de forma que sean aptos para su consumo según la normativa vigente y las condiciones reflejadas en el etiquetado de los mismos.

Los SGSA se componen de una serie de normas y principios, tales como el Análisis de Peligros y Puntos de Control Crítico (APPCC) cuya implantación en la agroindustria (de forma simplificada o completa) es de obligado cumplimiento según Reglamento nº 852/2004, que establece los principios de higiene de los productos alimenticios. El sistema APPCC se conoce como un procedimiento sistemático y preventivo, reconocido a nivel internacional, que evalúa la presencia y severidad de peligros de tipo biológico, químico y físico por medio de principios armonizados. Con este enfoque trata de evitar el análisis exclusivo de los productos finales y en su lugar, trata de determinar las causas por las cuales puede producirse una contaminación en los alimentos (Figura 1). El establecimiento de puntos críticos de control permite identificar el origen de dicha contaminación y evitar que se produzca, o en caso de haberse producido, establecer las correspondientes medidas correctoras. En el sector de la Aceituna de Mesa, existe la guía de gestión de la calidad de la industria de la aceituna de mesa (COI, 2005) que recoge los puntos que se deben de incluir en un sistema de APPCC en las empresas que elaboran aceitunas de mesa.

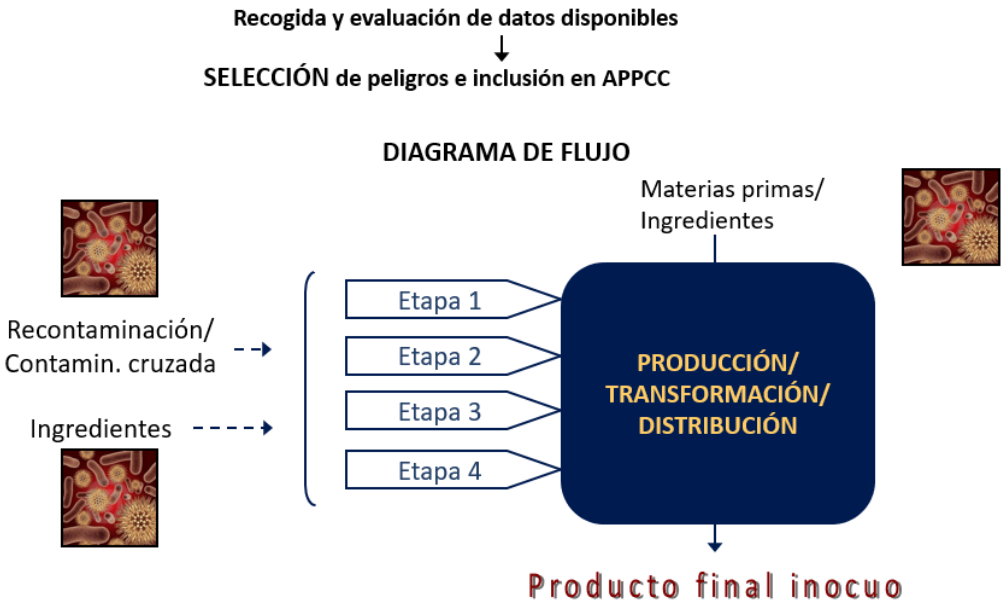

Figura 1. Diagrama de procesos para la identificación de peligros durante la producción de aceitunas de mesa.

El enfoque preventivo está basado en controles sobre la higiene del proceso a lo largo de las diferentes etapas del mismo. Por tanto, el sistema APPCC junto con la aplicación de Buenas Prácticas de Fabricación (BPF) son herramientas de gran utilidad para que el producto final cumpla con las especificaciones higiénicas. El plan APPCC resultante puede integrarse dentro de un sistema más general de aseguramiento de la calidad. En su forma más simple, se compone de 7 principios generales:

- Principio 1. Realizar un análisis de peligros
- Principio 2. Determinar los puntos críticos de control (PCCs)
- Principio 3. Establecer límites críticos
- Principio 4. Establecer un sistema de vigilancia
- Principio 5. Establecer las medidas correctoras que habrán de adoptarse cuando la vigilancia en un PCC indique una desviación respecto a un límite crítico establecido
- Principio 6. Establecer procedimientos de verificación para confirmar que el sistema APPCC funciona eficazmente
- Principio 7. Establecer un sistema de documentación sobre todos los procedimientos y los registros apropiados para estos principios y su aplicación

Además de los beneficios anteriormente reseñados, la correcta aplicación de los sistemas APPCC pueden ayudar a obtener una mejor gestión de los recursos disponibles por parte de la empresa lo cual supone un ahorro económico a la vez que un aumento de la competitividad de la misma. Otro beneficio derivado de su aplicación reside en los manipuladores de alimentos. Si asumimos que el principal capital con el que cuenta la empresa es el conocimiento y la destreza de su equipo humano, el que las personas adquieran conocimientos y una mayor concienciación sobre la importancia del cumplimiento de las normas de higiene durante el proceso de elaboración, dando como resultado una mejora de las prácticas de manipulación y evitando una posible contaminación de los alimentos, tiene como consecuencia directa, un mayor crecimiento profesional del equipo e incide directamente sobre la motivación del mismo.

Por parte de las autoridades gubernamentales y organismos de control oficial, el sistema APPCC proporciona un mayor nivel de confianza sobre la salubridad de los alimentos para la población consumidora, reduciendo notablemente la probabilidad de alertas a la vez que se facilita su comercialización a nivel internacional.

En relación con el sector de la aceituna de mesa, las elaboraciones tradicionales involucran una serie de etapas complejas donde tienen lugar multitud de procesos a nivel biológico y físico-químico, ya que lo que se persiguen son alimentos fermentados. Esto da lugar a que, en algunos casos, haya una falta de estandarización en los procesos, lo cual dificulta la comercialización del producto final o la apertura de nuevos canales, puesto que las tendencias del mercado están encaminadas hacia la homogenización del producto final.

Existen en la bibliografía algunos estándares como el CODEX Alimentarius francés (CODEX, 2000) sobre los criterios y limites microbiológicos que la aceituna de mesa debe cumplir. El documento CODEX estándar (CODEX STAN 66- 1981, revisado en 1987 y 2013) así como el documento que dicta los requisitos de comercialización de las aceitunas de mesa (International Olive Oil Council, IOOC, 2004) recomiendan que las elaboraciones de aceitunas de mesa deben prepararse de acuerdo con los principios generales de higiene del CODEX Alimentarius (CAC/RCP 1- 1969), el código de prácticas higiénicas para alimentos acidificados y conservas de baja acidez (CAC/RCP 23- 1979) y el código de prácticas higiénicas para conservas de fruta y alimentos vegetales (CAC/RCP 2- 1969). La normativa

europea aplicable a la higiene de los alimentos se resume en el Reglamento (CE) nº 852/2004, del Parlamento Europeo y del Consejo, relativo a la higiene de los productos alimenticios, establece que las empresas alimentarias deben cumplir con el requisito obligatorio de elaborar, aplicar y mantener un procedimiento permanente basado en los principios del sistema APPCC.

Además de la normativa aplicable, existen en la literatura otras guías procedentes del Consejo Oleícola Internacional (COI, 2004), así como de organizaciones sectoriales como ASEMESA (www.asemesa.es). Sin embargo, se reconoce que, por una parte, existe una amplia variedad de situaciones en las empresas de la cadena alimentaria y de productos ofrecidos al consumidor, y por otra, es necesario establecer mecanismos de flexibilidad para que los procedimientos de autocontrol puedan aplicarse de una forma general en todas las situaciones, incluidas las pequeñas empresas, las cuales tienen una baja facturación y un número de trabajadores reducido, en muchos casos, inferior a las 15 - 20 personas, por lo cual se dificulta la asignación de recursos, ya sean económicos o técnicos a la vez que personal especializado.

Son muchos los obstáculos que se presentan a las pequeñas empresas en las fases iniciales de aplicación del sistema APPCC, tanto de tipo administrativo como de tipo técnico. Como se ha dicho anteriormente, uno de los principales motivos que limita la aplicación de este sistema en las pequeñas empresas, es la dificultad de inversión, a lo que se le une el desinterés provocado por la idea que, conociendo el sector, puede percibirse por parte del industrial sobre la innecesaria instauración de nuevas medidas de control sobre un producto archiconocido para ellos, sobre los que tienen una gran experiencia en su manejo, en muchos casos, cuentan con varias generaciones y un difícil retorno del esfuerzo invertido en forma de ingresos. Tanto es así, que el cumplimiento de la norma, en muchos casos, solo tienen un único retorno, evitar una sanción económica de la administración sanitaria competente.

Las empresas de menor tamaño difícilmente cuentan con suficientes recursos como para contratar profesionales o asesores externos, o no quieren destinarlos a estos menesteres, por las razones anteriormente argumentadas. El tiempo necesario para que una pequeña empresa desarrolle un plan de seguridad de alimentos oscila entre los tres meses y algo más de un año en condiciones normales. En la mayoría de los casos, si existe una asociación de

productores, es frecuente que sea esta quién contrate a asesores o especialistas que tiendan a elaborar un programa tipo, aplicable a la mayoría de las situaciones, aunque suelen requerir de adaptaciones a las circunstancias de cada empresa, algo que, en muchos casos, no se realiza derivándose consecuencias no siempre positivas a raíz de las inspecciones de las autoridades sanitarias. En este sentido, la creación de estas asociaciones, es decir la actuación de las pequeñas empresas como bloques con objetivo común, facilita el desarrollo de los sistemas APPCC, aunque solo sean para alcanzar un cumplimiento legal, dejando de lado los posibles retornos empresariales que podría tener el adecuado manejo de la información que se ha de generar obligatoriamente.

A lo largo de la cadena de elaboración de aceitunas de mesa, hay ciertos aspectos que deben tenerse en cuenta y que deben incluirse en los sistemas APPCC, entre otros:

a) Calidad higiénica de las materias primas, que puede verse afectada si las aceitunas son recogidas directamente desde el suelo
b) Prácticas de manipulación durante la producción primaria, así como en el momento de la recepción de la materia prima
c) Estado higiénico -sanitario de superficies de trabajo
d) Condiciones del proceso de fermentación (acidez, pH, NaCl etc.)
e) Adición de aliños (hierbas o especias)
f) Calidad sanitaria del agua utilizada para el lavado y la adición de salmuera
g) Peligros de origen químico (sustancias tóxicas) o físicos (trazas de metales o cristal) potencialmente presentes en el producto final
h) Condiciones de envasado, almacenamiento y distribución.

Para la producción de aceitunas de mesa, las etapas del proceso que deben incluirse en los sistemas APPCC están asociadas a la región donde se producen o a los procesos de elaboración tradicionales que se siguen, ya que, como el caso que nos ocupa, son preparaciones muy arraigadas en un territorio con ciertas peculiaridades. En líneas generales, todos ellos se componen fundamentalmente de las etapas de recepción de materias primas, procesos de fermentación, calibrado, lavado y partido (en su caso), cocido, tratamientos térmicos, adición de especias (en su caso) envasado y almacenamiento.

Para la identificación de peligros se requiere del conocimiento exhaustivo del proceso de elaboración, así como del producto final a elaborar. De forma general, se tendrán en cuenta los peligros potenciales que pueden aparecer en las etapas del proceso estableciendo una priorización en función de su probabilidad de aparición y severidad.

La definición de PCCs debe hacerse siempre y cuando sean etapas esenciales para prevenir y/o eliminar el peligro o reducirlo hasta niveles aceptables. Es habitual en esta etapa utilizar árboles de decisión, aunque puede que no sea aplicable en todas las situaciones. Generalmente, se requiere de un periodo de formación de equipo APPCC para la correcta definición de los PCCs del proceso. Posteriormente, deben establecerse límites críticos definidos como los valores mínimos o máximos del peligro que pueden tolerarse en una determinada etapa. Para establecer los límites críticos debe procederse a una revisión de la normativa vigente, así como revisión bibliográfica y estudios de validación del proceso en cuestión. A modo de ejemplo, en la Tabla 2 se señala la información referente a la fase de envasado de aceitunas de mesa que podría formar parte de un sistema APPCC. En ciertos casos, se determinan unos márgenes de tolerancia para cada límite crítico. En el caso de los parámetros físico-químicos, las directrices que marca el CODEX Alimentarius para aceitunas de mesa recomiendan una serie de límites mínimos de NaCl (5-6%) y máximos de pH (4,3) en los envasados en el caso de aceitunas verdes naturales, pasteurizadas y negras oxidadas, así como para aceitunas deshidratadas.

En relación con los criterios microbiológicos, las aceitunas de mesa están catalogadas como alimentos listos para el consumo y, por tanto, se acogen a la normativa europea vigente (Reglamento (CE) nº 1441/2007) por la que se establecen criterios microbiológicos en productos alimenticios. En función de la formulación del producto (condiciones de pH y a_w) las aceitunas de mesa pueden o no tolerar el crecimiento de *Listeria monocytogenes* según lo dictaminado en el anexo II del anterior Reglamento (pH < 4.4 o a_w < 0.92). En caso de que pueda tolerarse el crecimiento de *L. monocytogenes*, se admite un criterio de 100 UFC/g antes del consumo si el industrial puede demostrar frente a la autoridad competente que no se supera dicho nivel a lo largo del almacenamiento. En caso contrario, el criterio a aplicar será de ausencia en 25g.

Otras recomendaciones generales (COI, 2004) establecen que, aplicando procedimientos de muestreo y análisis apropiados, las aceitunas de mesa no deben presentar ningún tipo de contaminante, o en su caso, deben estar por debajo de los límites que puedan comprometer la salud de los consumidores. Las recomendaciones del CODEX Alimentarius francés (CODEX, 2000) marcan limites más específicos para aceitunas de mesa (microorganismos esporulados, *Staphylococcus* coagulasa positivos, anaerobios, bacterias ácido-lácticas o mohos y levaduras entre otros). En cualquier caso, dichos criterios pueden sufrir variaciones en función del tipo de aceituna y proceso de elaboración.

Tabla 2. Ejemplo de información a incluir en un sistema APPCC en la fase de envasado de aceitunas de mesa

Etapa del proceso: Envasado	
Peligro	Crecimiento microbiano indeseable debido a prácticas inadecuadas de almacenamiento Contaminantes de origen físico-químico y microbiológico en el envasado y producto final (fruto y salmuera)
Límites críticos	pH < 4.3 Concentración de NaCl > 6%
Monitorización y frecuencia de actividades	Chequeo periódico de los niveles de pH y NaCl en los envasados Chequeo para verificar los niveles de salmuera
Acciones correctoras	Acidificación y/o adición de NaCl Re-establecimiento de BPF
Responsable de las actividades de monitorización y medidas correctoras	Operador/supervisor
Documentación y control	Resultados de inspección de control oficial Resultados analíticos Acciones correctoras

Por último, en relación a este punto, es importante señalar que los sistemas APPCC en empresas elaboradoras de aceituna de mesa deben adaptarse a cada tipo de empresa y elaboración. En cada caso, es importante establecer las causas por las cuales la calidad y/o seguridad del producto final puede verse comprometida. En la Tabla 3 se detallan dichas causas y posibles medidas correctoras y/o de control.

Tabla 3. Posibles deficiencias y medidas correctoras y/o de control en las etapas del proceso de elaboración de aceituna de mesa

MOTIVOS que comprometen la calidad y/o seguridad del producto	**MEDIDAS CORRECTORAS Y/O DE CONTROL**
Alta carga de microorganismos en las muestras ambientales	- Equipos de ozonización - Ventiladores y filtros de aire Lámparas UV de activación nocturna / Diseño de barreras, puertas móviles, lamas, etc. separando las distintas etapas del proceso total para evitar la contaminación del ambiente.
Superficies de trabajo con alta carga microbiana	- Limpieza diaria con agua a presión caliente (70ºC) - Lámparas UV de activación nocturna - Uso de desinfectantes y agua bien clorada
Aliños con alta carga microbiana de partida (especialmente ajos y hierbas aromáticas)	- Escaldado o esterilización de los aliños - Sustitución o/y complementar con el uso de aromas o aceites esenciales.
pH de envasado excesivamente alto	- Bajada de pH con la adición de ácidos orgánicos
Condiciones higiénicas deficientes en determinadas etapas del proceso (ej. partido)	- Empleo de duchas dinámicas con agua clorada y realizadas durante la fase de transporte en cinta. - Planes de formación del personal - Uso de indumentaria adecuada; guantes, batas, botas, mascarillas, etc.
Mejor regulación del pH en el producto envasado	Disposición en la etapa de envasado de un pH metro para un mejor control de este parámetro de forma rutinaria en el producto ya terminado.

De lo anteriormente señalado, se destaca la importancia de implementar los SGSA en el sector de la aceituna de mesa. Algunos aspectos se recogen en la norma de calidad que ha sido recientemente publicada (RD 679/2016). Tal y como se señala en la normativa, existe una necesidad de responder a las demandas realizadas por el sector sobre una revisión de la misma enfocando la adaptación a las exigencias de los consumidores, del mercado y al

desarrollo tecnológico e innovación en lo que a la recolección y procesado se refiere, todo ello para asegurar la competitividad del sector de la aceituna de mesa española a nivel global. La norma de calidad incluye información sobre los procesos básicos de elaboración, formas de presentación, ingredientes, características de los productos terminados, defectos, categorías comerciales, peso neto escurrido mínimo e información facilitada al consumidor.

Sin embargo, según el enfoque seguido en el Plan Nacional de Control Plurianual de alimentos (PNCPA) (Reglamento (UE) 2017/625) la evaluación de los procedimientos de buenas prácticas de fabricación, prácticas correctas de higiene, y buenas prácticas agrícolas entre otros, deben basarse en los principios del Análisis de Riesgos y Puntos de Control Críticos (ARPCC). El sistema ARCPC identifica peligros específicos (físicos, químicos y biológicos) y medidas de control con el fin de asegurar la seguridad de los alimentos. El ARCPC es una herramienta para evaluar peligros y establecer sistemas de control enfocados en la prevención, en sustitución del análisis del producto final. El sistema ARCPC es capaz de adaptarse a situaciones cambiantes, tales como adelantos en el diseño de los equipos, cambios en los procedimientos de procesado o nuevos desarrollos tecnológicos. Este sistema puede aplicarse a lo largo de la cadena alimentaria desde el productor primario al consumidor final. Una vez instaurado, el ARCPC es un sistema de gestión para asegurar la seguridad de los productos preparados en un establecimiento alimentario.

La aplicación del ARCPC es compatible con la implementación de sistemas de gestión de la calidad (i.e. ISO 9000) y es el método de elección en la gestión de seguridad alimentaria dentro de dichos sistemas. Aunque el ARCPC está destinado al control de la seguridad, sus principios pueden aplicarse a otros riesgos, como la prevención de fraudes en relación con el etiquetado, categorización, pesos, etc., u otros aspectos de calidad alimentaria. La implantación de los sistemas ARCPC exige la adaptación de los SGSA con el fin de que otorguen una mayor flexibilidad y puedan, por tanto, adaptarse a las necesidades del sector. Estos enfoques podrían estar basados en sistemas de cuantificación que faciliten a la industria datos acerca de las condiciones higiénico-sanitarias con las que cuenta a lo largo del proceso, desde la recepción de la materia prima hasta el envasado y comercialización del producto final. Estos sistemas conforman una herramienta de considerable utilidad tanto para las empresas e industrias como para las autoridades sanitarias. Todo ello con un doble objetivo, por una parte, la mejora de la calidad del producto que se ofrece al consumidor mediante la

aplicación de medidas preventivas o en su caso correctoras y, por otra parte, la búsqueda de posibles ampliaciones de este sector mediante la distribución de este producto único y especial, aunque cada vez más extendido a nivel global, hacia nuevos mercados, proporcionando una revalorización social y económica de los territorios donde se cultiva el olivar y existe una economía vinculada al mismo.

Durante la realización de los trabajos de campo que han dado como resultado las distintas publicaciones recogidas en la presente Tesis Doctoral, se ha desarrollado un sistema de toma de decisiones basado en un enfoque preventivo que tiende a evaluar la importancia relativa de cada fase del proceso de elaboración de la aceituna *Aloreña de Málaga* proporcionando unas estimaciones basadas en un nivel de cumplimiento final, de ahí la importancia de elaboraciones minoritarias para la elaboración de modelos que puedan ser extrapolados a otras preparaciones con mayor grado de industrialización/innovación. Tanto es así, que este modelo se ha adaptado a la aceituna elaborada según el "estilo español" y ha servido para el desarrollo de un software con el que operar de forma fácil, ágil y cómoda en la agroindustria, empleando para ello el trabajo/esfuerzo que se debe realizar para dar cumplimiento a la normativa vigente sobre seguridad alimentaria.

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2. HIPÓTESIS Y OBJETIVOS DE TRABAJO

La presente Tesis Doctoral surge de la necesidad del sector de modificar, mejorar, modernizar e innovar en el proceso de transformación de la aceituna de mesa con DOP *Aloreña de Málaga* para responder a las actuales demandas del mercado y obtener un producto con los más altos estándares de calidad y seguridad alimentaria. Para este fin, partimos de las siguientes hipótesis de trabajo:

i) La aplicación de nuevas metodologías científicas en el sector (ómicas y modelado estadístico) proporcionaran una mayor información de los procesos de fermentación y conservación que ayudarán en la posterior toma de decisiones de cara a mejorar la calidad y seguridad del producto.

ii) Es posible una modificación del proceso fermentativo para la obtención de frutos con un menor contenido en cloruro de sodio y una mayor retención del color verde de las aceitunas y estabilidad de los envasados.

iii) Las diferentes etapas del proceso de transformación y el sistema APPCC en las industrias del sector puede ser mejorado y cuantificado mediante modelos matemáticos de riesgo.

Para confirmar estas hipótesis de trabajo, se han planteado los siguientes objetivos específicos:

1) Aplicación de un modelo matemático ADF para el estudio y modelado de los datos físico-químicos y microbiológicos obtenidos durante la etapa de fermentación y conservación de aceitunas tradicionales y curadas *Aloreña de Málaga* con bajo contenido en cloruro de sodio y con un periodo de reposo de los frutos previo a su puesta en salmuera. Este objetivo se ha abordado a través de los Capítulos 1 y 2.

2) Aplicación de técnicas de secuenciación masiva (metagenómica) para el estudio de la evolución de las poblaciones microbianas (fúngicas y bacterianas) durante el proceso fermentativo de aceitunas tradicionales *Aloreña de Málaga*. Este objetivo se ha abordado a través de los Capítulos 3 y 4.

3) Aplicación de tratamientos térmicos a los frutos de aceituna *Aloreña de Málaga* previo a su colocación en salmuera con la intención de favorecer la fermentación láctica, retención del color verde de los frutos y estabilidad de los envasados. Este objetivo se ha abordado a través del Capítulo 5.

4) Recogida de datos físico-químicos y microbiológicos para el estudio de los actuales sistemas de APPCC en las principales industrias del sector para el desarrollo de procedimientos científicos que permitan una mejora en el tratamiento de la información y gestión de la calidad y seguridad alimentaria. Este objetivo se ha abordado a través del Capítulo 6.

3. RESULTADOS

3.1 SECCIÓN I

3.1.1. CAPÍTULO 1

Assessment of table olive fermentation by functional data analysis

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Assessment of table olive fermentation by functional data analysis

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ABSTRACT

For the first time, functional data analysis (FDA) was used to assess the effects of different treatments on Protection Denomination of Origin *Aloreña de Málaga* table olive fermentations, focusing on the evolution of yeast population. The analysis of fermentation by a conventional approach led to scarce information. However, the transformation of microbial (and also physicochemical) data into smooth curves allowed the application of a new battery of statistical tools for the analysis of fermentations (functional pointwise estimation of the averages and standard deviations, maximum, minimum, first and second derivatives, functional regression, and functional F and t-tests). FDA showed that all the treatments assayed led to similar trends in yeast population while changes in pH and titratable acidity profiles led to several significant differences. Therefore, FDA represents a promising and valuable tool for studying table olive fermentations and for food microbiology in general.

1. Introduction

Table olive fermentation, one of the most important fermented vegetables of the Mediterranean basin, implies changes in several physicochemical and microbiological parameters over time (Garrido-Fernández et al., 1997). Usually, these changes are monitored, and the data subjected to diverse statistical approaches, particularly the fitting of appropriate kinetic models (Arroyo-López et al., 2010). Then, the effects of the environmental variables on the estimated parameters are analysed by ANOVA, Response Surface Methodology, logistic regression, or multivariate methods (Arroyo-López et al., 2010; Bautista-Gallego et al., 2013a; Bevilacqua et al., 2009). Specifically, for the study of microbial populations during fermentation of *Aloreña de Málaga*, a table olive speciality with Protection Denomination of Origin (PDO) in Spain, several primary and secondary predictive models have been applied. This way, the microbial decay and growth data were modelled by Weibull survival and Gompertz models, respectively (Arroyo-López et al., 2007). A quasi-chemical primary model fitted lactic acid bacteria (LAB) and yeast populations during *Aloreña de Málaga* storage not only during growth but also throughout the declining phase (Echevarria et al., 2010). The physicochemical parameter changes occurring during olive fermentation can also follow diverse kinetic models. Pseudo-second and first order decay models fit the changes in sugars, polyphenols, and colour from cracked *Aloreña de Málaga* olives (Arroyo-López et al., 2007, 2008; Bautista-Gallego et al., 2011) while a third-order kinetic model fits the lactic acid production in a mixture of diverse chloride salts (Bautista-Gallego et al., 2010). However, microbiological (or physicochemical) data obtained from fermentation processes, and especially from table olives, cannot always be satisfactorily fitted by mathematical models, a circumstance that could make the quantitative study of the process and the comparison among treatments difficult.

Although functional data models have been a rather common technique in statistics, the term functional data analysis (FDA) was popularized by Ramsay and Dalzell (1991). FDA is related to the representation, summarization, and analysis of data obtained from curves. Thereby, FDA considers the data obtained from a series of samples over time as a curve or function. The concept is, then, clearly applicable to table olive fermentation because the microbiological (or physicochemical) data from the successive samples may represent the actual curve of their changes over time. The theoretical and practical aspects of FDA may be found in Ramsay and Silverman (1997, 2002, 2005) and Ramsay et al. (2009). Furthermore, Ramsay et al. (2014) also implemented an R package ("fda") for the analysis of data. Their studies are complementary to those carried out by Ferraty and Vieu (2006) on non-parametric data analysis. In the field of food technology, Bi and Kuesten (2013) have applied FDA to investigate the sensory intensity-time data, and their work represents an invaluable contribution for the diffusion of this methodology. Thereby, this statistical tool has very diverse applications. It has been used to study the degradation by the liver of chylomicron remnants, which excess may contribute to atherosclerosis (Nuzzo, 2002), or to assess the effects of different rearing environments on a population of asymptotic growth curves of Brown Kiwi (Jones et al., 2009). FDA of variance (fANOVA) was applied to study the effect of cultivar origin and shelf-life exposure time on the NIR apple spectra

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(Bobelyn et al., 2010). Functional Principal Component Analysis (fPCA) applied to voltammetric data (and their first derivative) from urea and melamine-adulterated and non-adulterated milk samples allowed their correct classification by K-nearest neighbours (Hilding-Ohlsson et al., 2012). Ferraty et al. (2007) used a spectrometric data set (developed to control the fat content) for the factor-based comparison of groups while Aguilera et al. (2013) have reviewed the FDA of chemometric data (spectrum of meat or NIR spectrum) to predict the oil content in corn samples. However, FDA has not been yet applied to the study of the microbial populations during food fermentations. Therefore, the application of FDA to study table olive processing would represent an important step in extending its application to vegetable fermentation and also to food microbiology in general.

The aim of the present work was the application of FDA (vs. conventional approach) to investigate the changes in the yeast population, as well as to their associated physicochemical data, during the fermentation of PDO *Aloreña de Málaga* table olive subjected to diverse brining conditions.

2. Material and methods

2.1. Experimental design

The study was performed with *Aloreña de Málaga* fruits, harvested at the green ripe stage during the 2013/14 season (Valle del Guadalhorce, Málaga, Spain). For the experiments, 154 kg of whole fruits (cured type), or cracked olives (traditional type), were placed in 220 l containers (drums), where they were subjected to spontaneous fermentation after brining in 66 l of the following solutions: i) CC treatment (usual brine conditions of cured olives): 7 g/100 ml NaCl, 0.1 g/100 ml citric acid (CA), 0.5 g/100 ml acetic acid (AA); ii) CI treatment (highly acidified, cured olives): no salt, 0.1 g/100 ml CA, 1.6 g/100 ml AA; iii) CII treatment (moderately acidified, cured olives): no salt, 0.1 g/100 ml CA, 1.0 g/100 ml AA; iv) CT treatment (usual brine conditions of cracked, traditional olives): 11 g/100 ml NaCl solution, and v) RT treatment (fruits cracked after 72 h respiration at room temperature): brined in an 11 g/100 ml NaCl solution. All the treatments (5 fermentation systems) were run in duplicate. The containers were covered with their lids and stored at room temperature in the factory S.C.A. Copusan (Alozaina, Málaga, Spain). At different sampling times (1, 15, 38, 52, 80, 137, 250 and 380 days), 15 ml samples were aseptically withdrawn from the centre of both replicates of each treatment (drums) for their microbiological and physicochemical analysis. When necessary, the removed brine was replaced with the corresponding original brine. Then, the drums were covered again with the lid till the next sampling.

2.2. Microbiological analyses

Brine samples were diluted, if necessary, in a sterile saline solution (0.9 g/100 ml NaCl), and plated using a Spiral System model dwScientific (Dow Whitley Scientific Limited, England) on appropriate medium. *Enterobacteriaceae* were enumerated on VRBD (Crystal-violet Neutral-Red bile glucose)-agar (Merck, Darmstadt, Germany), LAB on MRS (de Man, Rogosa and Sharpe)-agar (Oxoid) with 0.02% sodium azide (Sigma, St. Louis, USA), and yeasts on YM (yeast-malt-peptone-glucose medium)-agar (Difco™, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulphate as selective agents for yeasts. The plates were incubated at 30 °C for 24 (*Enterobacteriaceae)* or 48 (LAB and yeasts) hours and counted using a Flash & Go (IUL, Barcelona, Spain) image analysis system. Counts were expressed as $\log_{10}$ cfu/ml.

2.3. Physicochemical analyses

The analyses for pH and titratable acidity in the cover brines were carried out using the standard methods developed for table olives (Garrido-Fernández et al., 1997) using a Titroprocessor mod 670 (Metrohm Instrument, Herisau, Switzerland).

2.4. Statistical analysis

For the analysis of the microbial populations by a conventional approach, the model of the two-term Gompertz equation proposed by Bello and Sánchez Fuertes (1995) for microbial growth/decline was used to fit the data of the yeast population changes over time. Similarly, several kinetic models were also checked for fitting the pH and titratable acidity changes throughout fermentation, being the three parameter formation kinetic model ($y = a + b(1 + e^{cx})$, where a stands for the intercept, b for overall change in the parameter during the process, and c the rate of change), the only that partially fitted the pH changes. The overall comparison among treatments was also achieved by analyzing the areas below the curves of the several fermentation parameters vs. time (Bautista-Gallego et al., 2010). Their values were estimated by integration using the SigmaPlot v. 11 software package (Systat Software, Inc.).

The FDA approach used in this work consisted of transforming the data into functional objects and smooth curves by using a smoothing spline estimator (S_λ) (a cubic spline with continuous first two derivatives) based on the minimization of:

$$\sum_{i=1}^{n} (y_i - s(t_i))^2 - \lambda \int s''(t)^2 dt$$

where s stands for the second derivative of spline function and λ (the smoothing parameter) is intended for maintaining a balance between closeness of fit to the data (first term) and the roughness (sudden local variation) penalty (second term). The presentation of the full data set is usually visualized by plotting them vs. time. This functional object may be used for a series of estimations (pointwise evaluation, maximum, minimum, area below the curves, or an average of treatments and their standard deviation) as well as for the calculus of their first and second derivatives (the speed and acceleration, respectively, at which the changes occur).

Another approach for transforming data into smooth curves is by regression that is a type of functional linear model where the independent variables are indicator variables conveying membership in a combination of factor levels:

$$y_i(t) = \beta_0(t) + \sum_{i=1}^{q} x_{ij}\beta_j(t) + \varepsilon_i(t)$$

where $y_i(t)$ is a functional response, the values of x_{ij} are either 0 or 1, $i = 1, 2, \ldots$ N for curves, $j = 1, 2 \ldots .q$ for groups or products and $\sum_{i=1}^{q} \beta_j(t) = 0$ for all t. As result of its application, the corresponding fitted curves are obtained as well as maxima, minimum, and derivatives. These results may be also used for the comparison of treatments by fANOVA, using permutation testing, which is performed by permutation of observations across groups. The percentage of repetitions in which the calculated values of F exceeded the Fs obtained from the original data, is the p-value under de null hypothesis. The output of the test includes the fitted curves, their maximum (and its corresponding time), the overall p-value of the permutation F-test, a plot of the observed pointwise statistic values and the 0.05 critical values. A functional permutation t-test, performed similarly to the F-test permits the pointwise comparison between two treatments. The FDA was achieved using the R routines and "fda" functions for R software v. 3.2.1 (https://www.r-project.org/) developed by Bi and Kuesten (2013). Therefore, those interested in FDA application are kindly referred to their R routines and tutorial.

The relationships among the several outputs obtained from PDO *Aloreña de Málaga* fermentation, using both the conventional and the FDA approaches, were studied by multivariate techniques, particularly the exploratory biplot (Gabriel, 1971), which allows displaying

graphical information on the links among and within both samples and variables.

3. Results and discussion

This work uses a mathematical approach for analyzing the microbial data obtained from several treatments intended for improving the fermentation process of cured and traditional Spanish PDO *Aloreña de Málaga* table olives. The cured olive process (CC, CI and CII treatments) consisted of fermenting whole untreated olives in brine. The procedure used currently (CC) was developed by García et al. (1992) and has been effective for decades. However, in the last years, there is an increasing demand to reduce the content of sodium in foods and fermented vegetables (Bautista-Gallego et al., 2013b). Therefore, evaluation of other fermentation systems without NaCl (CI and CII treatments) is necessary. On the other side, the traditional process (CT) is achieved with cracked olives, which use just brine (11% NaCl) as holding solution; its main problem is the high concentrations of residual sugars at packaging (Arroyo-López et al., 2007), which reduction after the storage phase is still a challenge. The effect of cracking and putting the olives in the brine just after picking vs. exposing the fruits to air for 72 h before cracking (for the partial consumption of sugars by respiration) was also included in the study. Thus, the experimental design permits the comparison between the curing process, the traditional and other alternative systems.

3.1. Conventional approach

The microbial fermentation process of this table olive speciality is far apart from the green Spanish-style (Garrido-Fernández et al., 1997). Regardless of brining conditions, the *Enterobacteriaceae* and LAB populations were below the detection limit (<1.3 $\log_{10}$ cfu/ml) in treatments CT and RT (cracked olives) but, although absent during most of the storage process, they were detected in treatments CC, CI, and CII (whole olives). Specifically, *Enterobacteriaceae* were found at the first sampling time at populations levels lower 3.0 $\log_{10}$ cfu/ml, while LAB were detected only in the last sampling time at <6.0 $\log_{10}$ cfu/ml. The inhibition of *Enterobacteriaceae* may be due to the low initial pH in the acidified brines (Garrido-Fernández et al., 1997) while the LAB absence can be related to the presence of fairly high concentrations of polyphenols (Arroyo-López et al., 2007). In contrast, all the treatments showed the usual development of yeasts observed in this table olive speciality (Arroyo-López et al., 2007), which constitute, thus, the microbiological focus of this study.

The yeast population evolution during the fermentation did not show the typical growth/decline curve due to an intermediate valley (Fig. 1). This profile made that the model proposed by Bello and Sánchez Fuertes (1995), which was the best modeling option, fitted the yeast changes in only a few treatments but with hardly interpretable parameters (data not shown). In such circumstances, the area below the microbial growth/decline curve represents an approximation to the overall evolution of the microbial populations, as demonstrated previously (Bautista-Gallego et al., 2010) and, in this case, showed that their trends were quite similar since the one-way ANOVA applied to yeast areas did not show significant ($p = 0.056$) differences among them (Ruiz-Bellido et al., 2016).

Regarding physicochemical data, the pH increases in treatments CT and RT (traditional processes) were more rapid, due to the cracked condition of the olives, than in CC, CI, and CII (cured olives), which whole fruits caused a slower flesh/brine acid equilibrium. The model used for data fitting led to appropriate, although similar, results only when applied to cured (whole) olives. The different profiles were confirmed by the corresponding ANOVA applied to the areas under the pH curves, which showed significant ($p = 0.003$) differences in their pairwise comparisons. Changes in titratable acidity also showed characteristic profiles that could not be fitted by the usual kinetic models. The

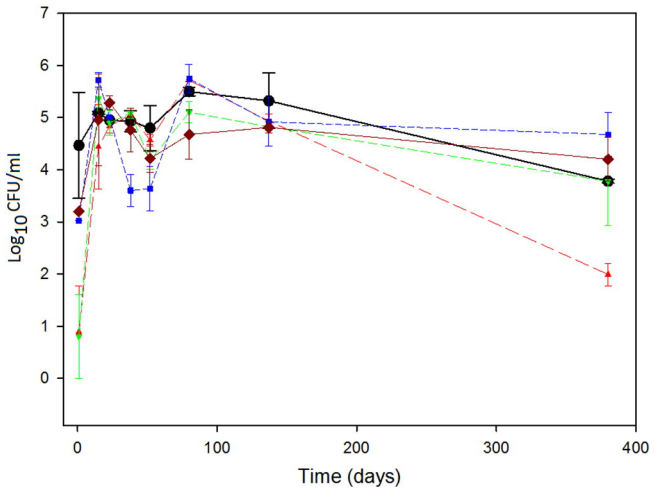

Fig. 1. Changes in the yeast population over time for all treatments assayed (—●— CC, —▲— CI, —▼— CII, —■— CT, and —◆— RT). CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate-acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

acidified treatments (CI and CII) followed particular trends, and the ANOVA applied to the areas under the titratable acidity curves showed significant overall ($p < 0.001$) and pairwise differences between them vs. the others. However, treatments RT vs. CT showed similar behaviors, indicating that, apparently, respiration at air for 2 days did not affect titratable acidity diffusion vs. the control (Ruiz-Bellido et al., 2016).

The overall comparison among fermentation systems, based on the area below the yeast, pH, and titratable acidity curves was achieved by PCA (Fig. 2). The first two PCs accounted for about 97% total variance; that is, they explained a considerable proportion of the total variability. The projection of the variables onto their plane showed that PC1 (F1) could be related to pH (negatively) and titratable acidity (positively); then, PC1 can be identified with the "physicochemical characteristics" and was very efficient segregating according to them. PC2 (F2) was linked to yeast population. The projections of cases showed that CI and CII were associated with a high acidity while CC and RT were linked to higher pH (Fig. 2). The position of the variable yeasts with respect to

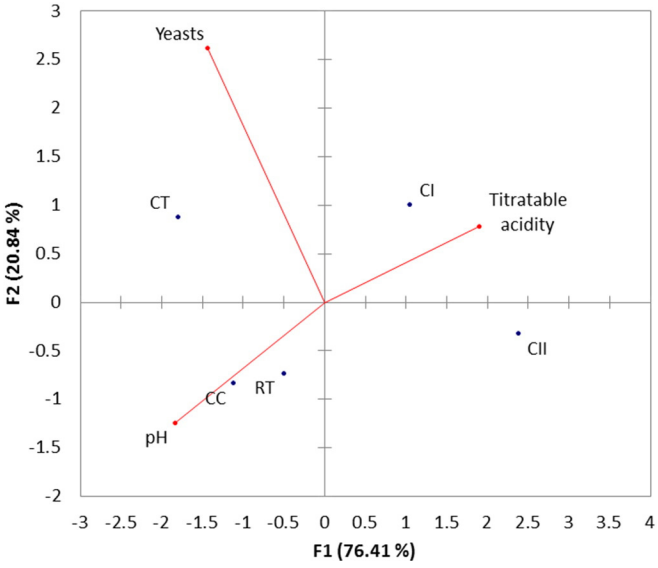

Fig. 2. Multivariate analysis for classical approach. Projection of variables and treatments on the plane formed by the first two principal components, based on the areas below the yeast population, pH, and titratable acidity. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate-acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

cases indicates that yeast population was mainly achieved in CT treatment while was lower and hardly influenced by the pH and titratable acidity of the brines (cosine of respective vectors ~90° ≅ 0). Therefore, the areas below the curves of the different parameters have been efficient for characterising and segregating treatments.

3.2. FDA approach

Due to the evident limitations showed by the classical approach for the quantitative study of this specific table olive elaboration, looking for new approaches seems pertinent. FDA, which has a complete battery of statistical tools for the comparison among treatments, is an attractive option. FDA allows the calculus of the averages and standard deviations of the area, maximum, minimum, speed and acceleration of changes by the application of smoothing and description of functional data objects, fANOVA, functional regression and functional F and *t*-tests using routines developed in R program. Below, we show only part of the information provided by this methodology. More information can be found elsewhere (Ruiz-Bellido et al., 2016).

The overall comparison among fermentation treatments over time was accomplished by combining the R code "fANOVA" and the built-in "fda" function "Fperm.fd". The overall trend was similar among all fermentation systems showing a first phase of active growth (during the first 20–30 days), a valley (around 50 days) and a slow declining of the population after the 100th day (Fig. 3, upper panel). The quantitative comparison of the yeast evolution in the diverse treatment was achieved by the "fANOVA", which overall permutation functional F-test led to a p-value = 0.04, indicating a global statistical difference between at least two treatments, although the pointwise graphical presentation of the F-values showed punctual significant differences only at ~30 (coinciding with the end of the first growth phase) and 120 (start of declining) days after brining (the highest population levels after the two periods of yeasts growth) (Fig. 3, lower panel). Therefore, in general, the treatments followed a quite statistically similar trend, with only limited effects at the times of maxima yeast populations. This overall comparison would not have been possible using the traditional approach.

The traditional approach for studying the microbiological changes is condensed into a few kinetic parameters which depend on the model used (Arroyo-López et al., 2007, 2010). However, the FDA approach also allows the quantification of the speed (rate of change) and acceleration (rate of change in speed) throughout the entire fermentation period, due to the continuous first and second derivatives, which have never been considered till now and are not usually provided when using the conventional approach. The speed (first derivative, related to growth/decline rates) and acceleration (second derivative, the rate of change) at which the microbial changes during fermentation occurred were obtained by the R code "fderiv". Speed (Fig. 4, panel A) and acceleration (Fig. 5, panel A) showed characteristic and well-differentiated changes during only growth time (up to ~100 days of fermentation, depending on treatments). The permutation functional F-test did not reveal significant differences in their speed (Fig. 4, panel B) or acceleration (Fig. 5, panel B) of yeast count changes (overall p-value = 0.41 and pointwise F-value always below the maximum and pointwise 0.05 critical values).

Regarding physicochemical data, the study of pH values by FDA showed that the diffusion of the acid into the flesh was slow (and, as a result, the brine pH increase was also slow) in all acidified treatments (CC, CI, and CII) while on the contrary, the cracking of the olive favoured a rapid acid equilibrium (and sharper pH increase) in the traditional olives (CT and RT). However, after ~150 days, the process led to a quite similar pH levels in all treatments. As a result, the overall permutation functional F-value resulted in significant ($p = 0.001$) differences between at least two treatments due to the pointwise F-values higher than the maximum and pointwise 0.05 critical value for the period during which the cracked olive curves were above the cured ones (~90 days). Therefore, the F-test confirmed that olive conditions (whole, in cured olives, vs. cracked, in traditional) led to significant differences in pH during the first brining period. Estimated titratable acidity over time yielded evident higher proportions in CI and CII treatments, in agreement with their initial proportions of AA, while the curves for CC were closer to those from traditional cracked olives. These consistent significant differences throughout the storage period (overall $p < 0.001$) led to permutation F-test values always above the maximum and pointwise 0.05 critical limits. Finally, the highest initial speed of changes in pH values corresponded to traditional olives (faster equilibrium in cracked olives than in whole olives), with scarce differences between freshly brined and olives allow respiration for 2 days. However, after ~75 days pH remains stable. The permutation functional F-test led to significant overall differences (overall p-value = 0.02), which was observed during the first 30–40 days (estimated F-values higher than maximum or pointwise 0.05 critical values). There was also significant differences in the speed of titratable acidity decrease (negative sign) in brine at selected periods of time (15–30 and 90–100 days), at which the estimated F-values were above the maximum 0.05 critical level. The eventual production of acid due to the growth

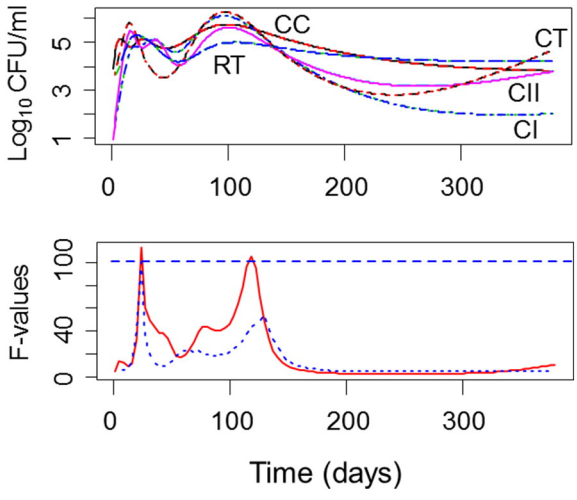

Fig. 3. Functional analysis of variance for the changes in yeast population (expressed as $\log_{10}$ cfu/ml) over time. Regression fitted curves for all treatments assayed (upper panel) and permutation F-test (lower panel), showing the observed pointwise F-values, together with its maximum (break line) and pointwise 0.05 critical values (dotted lines). CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate-acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

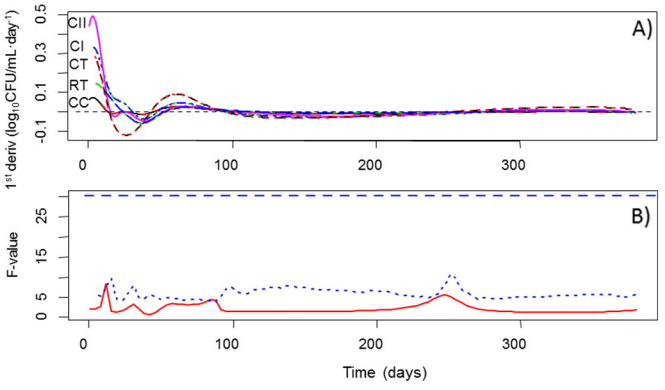

Fig. 4. Functional analysis of variance for first derivatives of yeast growth curves (panel A) and estimated permutation functional F-test for these curves (panel B) together with their maxima (broken lines) and pointwise (dotted line) p = 0.05 critical values. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate-acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

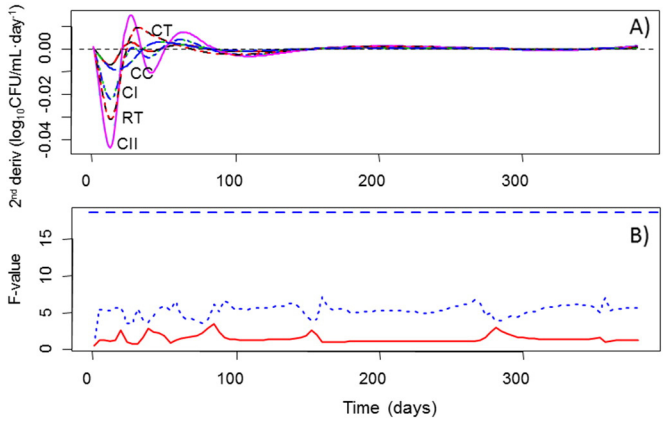

Fig. 5. Functional analysis of variance for second derivatives of yeast growth curves (panel A) and estimated permutation functional F-test for these curves (panel B) together with their maxima (broken lines) and pointwise (dotted line) $p = 0.05$ critical values. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate-acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

of LAB in some treatments by the end of the fermentation period might be associated with significant differences (p-values between the pointwise 0.05 critical values and the maximum limit). In general, the pointwise information allows detecting punctual effects that otherwise will be unnoticed (Nuzzo, 2002). The acceleration at which the changes in pH and titratable acidity occurred (as measured by the second derivative) showed systematic changes for ~90 days only. During this period, the accelerations were different among treatments only for pH. As in the traditional product, olive cracking facilitated the diffusion and the acceleration at which the changes of pH occurred. Later, the changes took place at null or very low speed. All this information can be extended elsewhere (Ruiz-Bellido et al., 2016).

The application of the "fANOVA" also estimated the corresponding maxima for the mean, speed and acceleration of yeast, pH and titratable acidity, according to treatments, and the times at which they were reached (Table 1). All of them should be, a priori, linked to the effect of fermentation systems or the environmental conditions. For example, the maximum yeast population was reached at around the 100th day of fermentation in most treatments; however, in RT, it was observed earlier (on the 21st day). Therefore, the previous exposure of fruits to air facilitated an easier nutrient release. Average maximum pH was reached faster in the traditional (cracked olives) process (CT, and RT) than in cured (not cracked) olives (CC, CI, CII) because the differences in diffusion rates.

Data from Table 1 were subjected to multivariate analysis. The biplot showed that the first two PCs explained a sensible proportion (80%) of the variance (Fig. 6). The PC1 (D1) could represent "time to reach maximum values", positively related to the time required to reach the maximum yeast counts (YTM), pH (pHTM), titratable acidity (ATM), and speed of acidity changes (A1DT), but negatively with the time to reach the maximum speed for pH changes (pH1DT). The PC2 (D2), might be identified with "acceleration" because its association with the greatest acidity (negatively) and yeast acceleration (positively) (A2DT and Y2DT respectively), which reproduces the usual negative correlation between pH and yeast counts in table olives (Garrido-Fernández et al., 1997). Also, the biplot showed a strong relationship among yeast population, acidity, and pH maxima, which reflects the fact that high pH and presence of an acid may contribute to yeast growth. The graph also showed the opposite relationship between RT treatment with time to reach maximum yeast counts (because the lower nutrient content of olives subjected to respiration), pH, and titratable acidity (due to the use of only salt in its brine), and the close relationship between CI and CII with the maximum acceleration in pH changes (due to their higher initial acidity). The biplot based on the data from the conventional approach (Fig. 2) was not efficient for grouping treatments prepared with whole olives (CC, CI, and CII); however, the biplot with data from FDA has produced a complete segregation between cured (whole) olives, on the right, and traditional (cracked), on the left. Furthermore, in this biplot, the PC2 was also able to segregate CC, cured control with low acidification, from CI and CII, highly and moderately acidified respectively, positioning them on the first and fourth quadrants, respectively. Therefore, the biplot with FDA data has been more realistic than that built with data from the conventional approach.

4. Conclusions

This work has applied, for the first time, the FDA to study the microbial evolution (and associated physicochemical data) of PDO *Aloreña de Málaga* olives subjected to diverse fermentation systems. Due to the peculiar characteristics of this processing, the information obtained using

Table 1
Maxima average values, speeds, and accelerations for the fermentation variables (yeast counts, pH, and titratable acidity) as well as the time at which they were observed, according to treatments. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate-acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

Variable/Treatment	Average maximum value		Maximum speed at which changes occur		Maximum acceleration at which changes occur	
	Time (d)	Value	Time (d)	Value	Time (d)	Value
Yeasts counts						
CC	99	5.7 ($\log_{10}$ cfu/ml)	3	7.2E−2 ($\log_{10}$ cfu/ml)d^{-1}	27	2.8E−3 ($\log_{10}$ cfu/ml)d^{-2}
CI	97	6.1 ($\log_{10}$ cfu/ml)	3	3.3E−1 ($\log_{10}$ cfu/ml)d^{-1}	61	4.0E−3 ($\log_{10}$ cfu/ml)d^{-2}
CII	101	5.6 ($\log_{10}$ cfu/ml)	3	4.9E−1 ($\log_{10}$ cfu/ml)d^{-1}	27	1.5E−2 ($\log_{10}$ cfu/ml)d^{-2}
CT	97	6.3 ($\log_{10}$ cfu/ml)	3	2.9E−1 ($\log_{10}$ cfu/ml)d^{-1}	32	9.5E−3 ($\log_{10}$ cfu/ml)d^{-2}
RT	21	5.3 ($\log_{10}$ cfu/ml)	3	1.5E−1 ($\log_{10}$ cfu/ml)d^{-1}	48	2.9E−3 ($\log_{10}$ cfu/ml)d^{-2}
pH value						
CC	270	4.5 (pH units)	3	8.7E−2 (pH units)d^{-1}	45	4.0E−4 (pH units)d^{-2}
CI	185	4.2 (pH units)	3	7.8E−2 (pH units)d^{-1}	104	1.0E−4 (pH units)d^{-2}
CII	206	4.3 (pH units)	4	7.6E−2 (pH units)d^{-1}	104	1.9E−4 (pH units)d^{-2}
CT	24	4.6 (pH units)	4	1.5E−1 (pH units)d^{-1}	41	2.6E−3 (pH units)d^{-2}
RT	23	4.7 (pH units)	4	1.65E−1 (pH units)d^{-1}	42	2.7E−3 (pH units)d^{-2}
Titratable acidity						
CC	380	1.0 (g/100 ml)	352	6.3E−3 (g/100 ml)d^{-1}	35	5.5E−4 (g/100 ml)d^{-2}
CI	380	2.4 (g/100 ml)	108	9.5E−3 (g/100 ml)d^{-1}	16	2.7E−3 (g/100 ml)$^{d-2}$
CII	380	2.0 (g/100 ml)	104	1.1E−2 (g/100 ml)d^{-1}	16	1.3E−3 (g/100 ml)d^{-2}
CT	3	0.6 (g/100 ml)	111	2.5E−3 (g/100 ml)d^{-1}	30	3.9E−4 (g/100 ml)d^{-2}
RT	1	0.6 (g/100 ml)	39	4.18E−3 (g/100 ml)d^{-1}	24	5.9E−4 (g/100 ml)d^{-2}

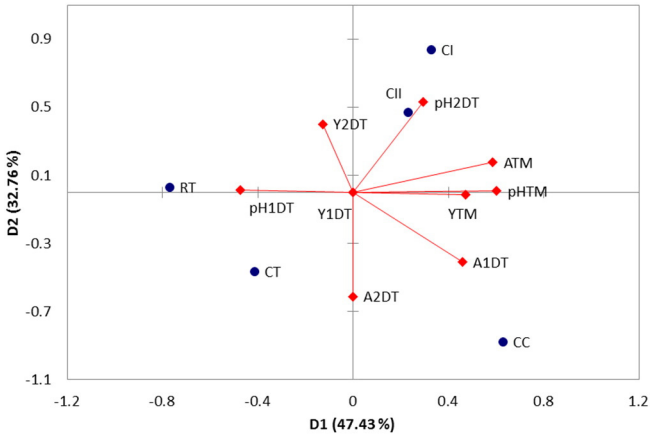

Fig. 6. Biplot for FDA data relating treatments with the time at which maximum values for means, first and second derivatives were estimated. Variables (loadings) and scores (treatments) projections on the plane of the first two PCs. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate-acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air. Y, pH, and A stands for yeast, pH and titratable acidity regression, respectively, while TM, 1DT and 2DT refer to the time to reach the maximum means as well as, first and second derivatives, respectively.

the conventional statistical approach was limited. On the contrary, FDA, based on smoothing or regression for the transformation of data into curves, was able to generate novel information (e.g. speed or acceleration at which the changes occur or overall/pointwise comparison among treatments), not usually provided by the conventional statistical analysis of the same data, and led to a better assessment of the treatments' effects. The promising results obtained in this work may contribute to extending the use of FDA not only to table olives (or vegetable fermentations) but also to other aspects of food microbiology.

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3.1.2. CAPÍTULO 2

Data on the application of functional data analysis in food fermentations

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Data Article

Data on the application of Functional Data Analysis in food fermentations

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ABSTRACT

This article refers to the paper "*Assessment of table olive fermentation by functional data analysis*" (Ruiz-Bellido et al., 2016) [1]. The dataset include pH, titratable acidity, yeast count and area values obtained during fermentation process (380 days) of *Aloreña de Málaga* olives subjected to five different fermentation systems: i) control of acidified cured olives, ii) highly acidified cured olives, iii) intermediate acidified cured olives, iv) control of traditional cracked olives, and v) traditional olives cracked after 72 h of exposure to air. Many of the Tables and Figures shown in this paper were deduced after application of Functional Data Analysis to raw data using a routine executed under R software for comparison among treatments by the transformation of raw data into smooth curves and the application of a new battery of statistical tools (functional pointwise estimation of the averages and standard deviations, maximum, minimum, first and second derivatives, functional regression, and functional *F* and *t*-tests).

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Specifications Table

Subject area	*Food Technology*
More specific subject area	*Microbiology, Statistics*
Type of data	*Tables, figures*
How data was acquired	*Use of a titroprocessor mod 670 (Metrohm Instrument, Herisau, Switzerland) for determination of pH and titratable acidity values. Use of a Spiral System model dwScientific (Dow Whitley Scientific Limited, England) for determination of yeast counts on selective medium.*
Data format	*Raw, analyzed*
Experimental factors	*Five fermentation systems of cured and cracked Aloreña olives with different NaCl and acidification conditions.*
Experimental features	*Monitoring of fermentations, microbial and physicochemical analysis, transformation of data into smooth curves, functional data analysis*
Data source location	*Alozaina, Málaga, Spain.*
Data accessibility	*Data available within this article*

Value of the data

- Use datasets as a benchmark for further functional data analysis or modelling of table olive fermentations.
- Application of functional data analysis for the study of food fermentations.
- Understand the influence of acidification and cracking of olives on the fermentation process of *Aloreña olives* by comparisons among different fermentation systems.

1. Data

The dataset provided in this article (Tables 1–7) and their corresponding Figures (Figs. 1–8) represent the raw microbiological (yeast counts) and physicochemical (pH and titratable acidity) data, as well as their statistical analysis by the application and implementation of Functional Data analysis, of different olive fermentation systems using *Aloreña de Málaga fruits*.

2. Experimental design, materials and methods

Olives were harvested at the green ripe stage during the 2013/14 season (Valle del Guadalhorce, Málaga, Spain) and subjected to five different fermentation system: i) CC (usual brine, control cured olives): 7 g/100 ml NaCl, 0.1 g/100 ml citric acid (CA), 0.5 g/100 ml acetic acid (AA); ii) CI (highly acidified, cured olives): no salt, 0.1 g/100 ml CA, 1.6 g/100 ml AA; iii) CII (moderately acidified, cured olives): no salt, 0.1 g/100 ml CA, 1.0 g/100 ml AA; iv) CT (usual brine of cracked, traditional olives): 11 g/100 ml NaCl solution, and v) RT (usual brine, olives cracked after 72 h respiration at room temperature): brined in a 11 g/100 ml NaCl solution. For the rest of the details of the experimental design, and how microbiological and physicochemical data were acquired, please consult the paper by Ruiz-Bellido et al. [1].

The Functional Data Analysis was achieved using the R routines and “fda” functions for R software developed by Bi and Keusten [2] and Ramsay et al. [3]. Therefore, those interested in its application are kindly referred to their R routines and tutorial. Please, consult also [1] for detailed information of how raw data were processed and analysed.

Table 1
Changes in yeast population (log_{10} cfu/ml) through the storage/fermentation process of Aloreña table olives. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

Time (days)	CC		CI		CII		CT		RT	
	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.
1	3.45	5.48	1.78	nd[a]	1.60	nd[a]	3.03	3.03	3.20	3.20
15	5.06	5.11	3.62	5.26	5.19	5.55	5.87	5.58	5.84	4.08
38	4.95	4.94	4.70	4.97	5.13	4.66	5.02	4.99	5.42	5.15
52	4.73	5.13	5.00	5.12	4.99	5.18	3.30	3.90	4.34	5.18
80	4.36	5.24	4.68	4.46	4.01	4.35	4.06	3.20	3.95	4.48
137	5.58	5.41	5.70	5.73	4.90	5.30	6.02	5.48	5.15	4.20
250	4.79	5.85	5.06	4.81	4.90	4.75	5.39	4.45	4.70	4.92
380	3.82	3.74	2.20	1.78	4.62	2.93	5.10	4.25	3.78	4.62

Repl. stands for replicate.

[a] nd, not detected (< 1.3 log_{10} cfu/ml).

Table 2
Changes in pH through the storage/fermentation process of Aloreña table olives. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

Time (days)	CC		CI		CII		CT		RT	
	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.
1	2.71	2.71	2.37	2.37	2.40	2.40	2.71	2.71	2.71	2.71
15	3.79	3.73	3.32	3.32	3.33	3.35	4.32	4.39	4.42	4.48
38	4.21	4.17	3.91	3.79	3.96	3.99	4.34	4.40	4.35	4.45
52	4.26	4.21	3.97	3.95	4.17	4.06	4.36	4.43	4.35	4.33
80	4.46	4.30	4.13	3.91	4.12	4.08	4.34	4.44	4.40	4.36
137	4.41	4.15	4.22	4.13	4.21	4.17	4.38	4.35	4.32	4.31
250	4.62	4.25	4.28	4.08	4.28	4.23	4.30	4.30	4.31	4.36
380	4.43	4.00	4.08	4.01	4.14	4.09	4.55	4.21	4.20	4.24

Repl. stands for replicate.

Table 3
Changes in titratable acidity (g lactic/100 ml brine) through the storage/fermentation process of Aloreña table olives. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

Time (days)	CC		CI		CII		CT		RT	
	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.	1st Repl.	2nd Repl.
1	0.61	0.61	2.40	2.40	1.60	1.60	0.61	0.61	0.61	0.61
15	0.49	0.49	1.59	1.87	1.26	1.33	0.49	0.49	0.44	0.44
38	0.37	0.38	1.53	1.50	1.20	1.18	0.40	0.40	0.4	0.31
52	0.43	0.43	1.55	1.48	1.12	1.14	0.43	0.43	0.41	0.41
80	0.49	0.47	1.51	1.37	1.06	1.12	0.39	0.41	0.39	0.34
137	0.77	0.81	1.91	1.92	1.68	1.59	0.54	0.53	0.54	0.54
250	0.44	0.71	1.75	2.02	1.47	1.55	0.46	0.50	0.44	0.38
380	0.94	1.11	2.36	2.45	1.89	2.01	0.43	0.59	0.53	0.48

Repl. stands for replicate.

Table 4
Average areas (± SE) below the yeast, pH and titratable acidity curves, according to treatments. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

Treatment	Yeast	pH	Titratable acidity
CC	1808 (64)	1618 (37)	253 (16)
CI	1505 (29)	1529 (17)	718 (13)
CII	1882 (76)	1553 (6)	576 (5)
CT	1820 (99)	1637 (6)	184 (5)
RT	1726 (34)	1627 (3)	171 (5)

Notes: One way ANOVA for the areas below the curves led to following p-values: 0.056, 0.003, and <0.001, for yeast, pH and titratable acidity, respectively.

Table 5
Changes in pH during storage/fermentation process of Aloreña table olives. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air. Parameters (± SE) of the model fit over time ($y=a+b(1-\exp(-cx))$).

Treatment	*a*	*b*	*C* (days^{-1})
CC	2.6 ± 0.1	1.8 ± 0.1	(8.6 ± 1.5)E-2
CI	2.2 ± 0.1	1.9 ± 0.1	(6.6 ± 0.7)E-2
CII	2.2 ± 0.1	2.0 ± 0.1	(6.9 ± 0.7)E-2
CT	2.0 ± 0.9	2.4 ± 0.9	(0.35 ± 0.38)*
RT*	--------*	--------*	--------*

a, intercept; *b*, overall change in pH; *c*, rate of pH change.

* Non-significant parameters.

Table 6
Pairwise comparison of pH values between the areas of the different storage/fermentation Aloreña table olive treatments (Fisher LSD method, ANOVA p-value=0.003). CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

Comparison	Diff of Means	LSD (alpha=0.050)	*P*	Diff ≥ LSD
CT vs. CI	108	68	0.009	Yes
CT vs. CII	84	68	0.024	Yes
CT vs. CC	20	68	0.489	No
CT vs. RT	10	68	0.728	No
RT vs. CI	99	68	0.013	Yes
RT vs. CII	74	68	0.037	Yes
RT vs. CC	10	68	0.721	No
CC vs. CI	89	68	0.020	Yes
CC vs.CII	64	68	0.059	No
CII vs. CI	25	68	0.396	No

Table 7
Pairwise comparison of titratable acidity values between the areas of the different storage/fermentation Aloreña table olive treatments (Fisher LSD method, ANOVA p-value < 0.001). CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

Comparison	Diff of Means	LSD ($\alpha = 0.050$)	*P*	Diff $\geq$ LSD
CI vs. RT	547	40	< 0.001	Yes
CI vs. CT	534	40	< 0.001	Yes
CI vs. CC	465	40	< 0.001	Yes
CI vs. CII	142	40	< 0.001	Yes
CII vs. RT	405	40	< 0.001	Yes
CII vs. CT	393	40	< 0.001	Yes
CII vs. CC	323	40	< 0.001	Yes
CC vs. RT	82	40	0.002	Yes
CC vs. CT	70	40	0.005	Yes
CT vs. RT	12	40	0.427	No

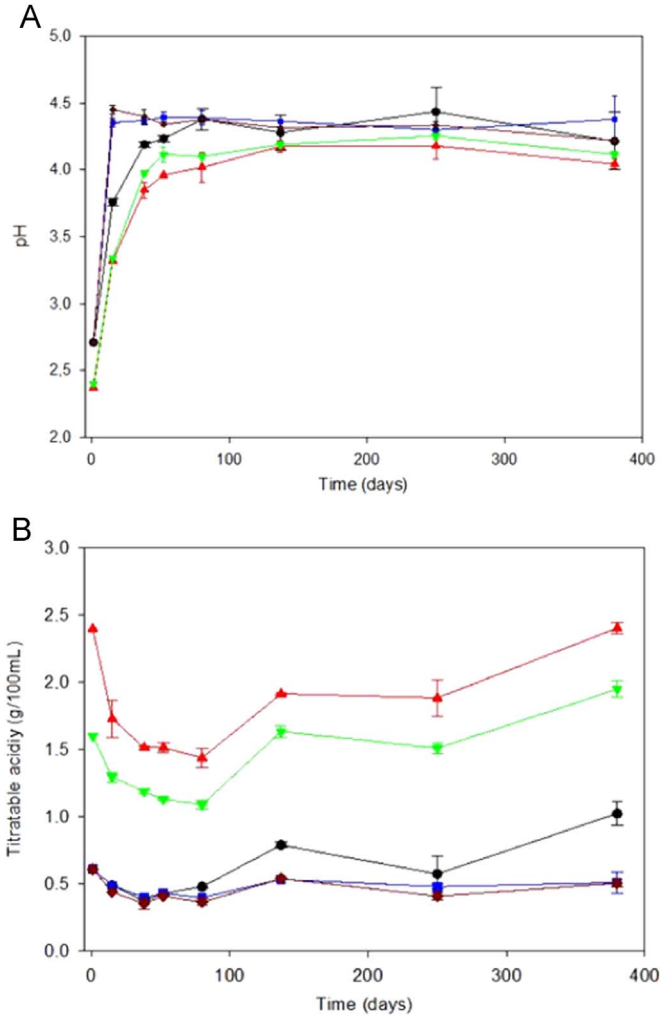

Fig. 1. Changes in pH (panel A) and titratable acidity (panel B) over time, according to treatments (CC, CI, CII, CT and RT). CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

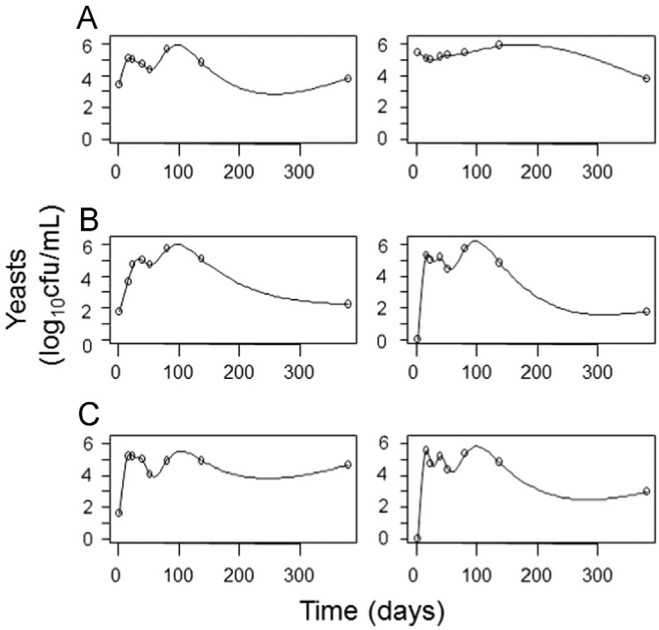

Fig. 2. Graphical presentation of some examples of yeast population smoothing; each row shows the two replicate of treatments CC (panel A), CI (panel B) and CII (panel C). CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives.

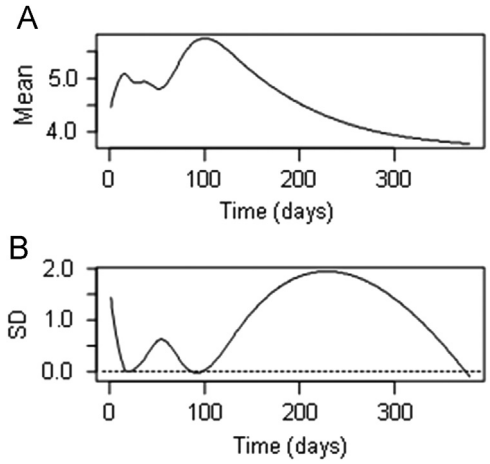

Fig. 3. Estimations of the average mean (panel A) and standard deviation (panel B) yeast in treatment CC, expressed as $\log_{10}$ cfu/ml, based on the yeast functional object obtained from smoothing. CC, control of acidified cured olives.

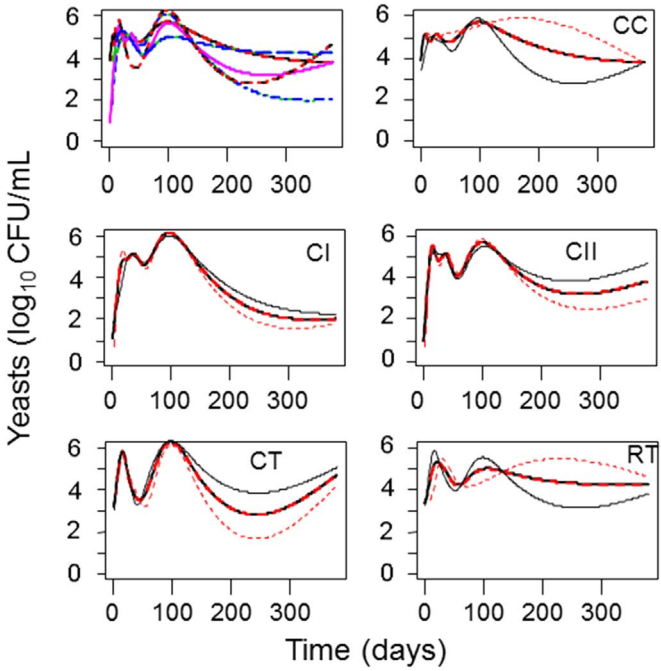

Fig. 4. Functional regression, showing the overall trends obtained for all treatments assayed (top left), followed by the average (and their replicates) of the specific profiles for each of the treatments. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

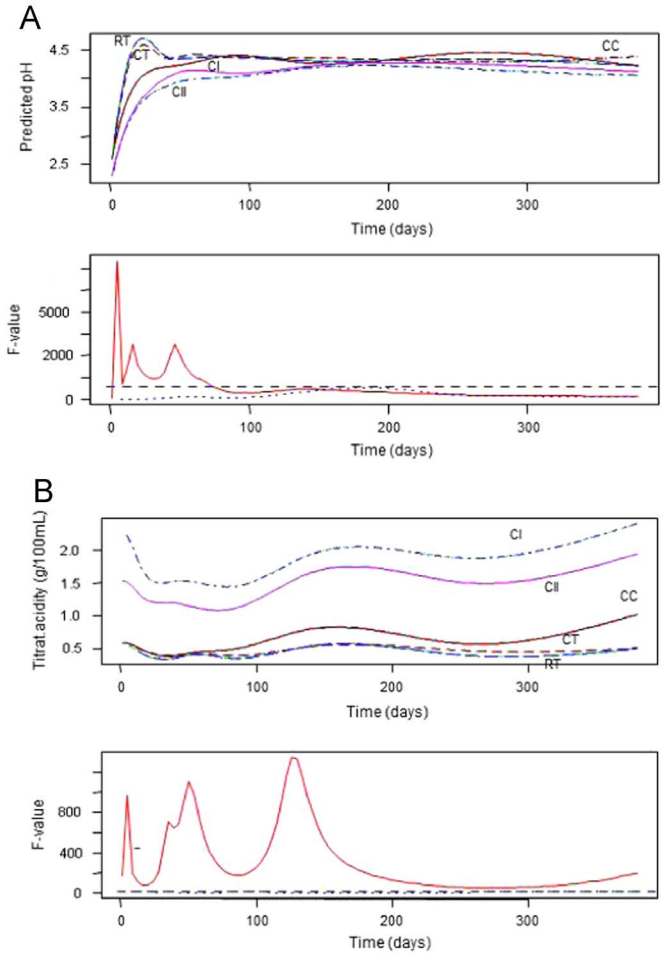

Fig. 5. Functional analysis of variance for the changes in pH (panel A) and titratable acidity (panel B) over time. Panel A: upper graph, predicted pH regression curves for the treatments assayed; bottom graph, pH permutation *F*-test for the curves above. Panel B: upper graph, regression predicted titratable acidity curves for the treatments assayed; bottom graph, permutation *F*-test for the above curves. In both permutation tests, the graphs show the observed *F*-value, together with its maximum (break line) and pointwise 0.05 critical values (dotted lines). CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

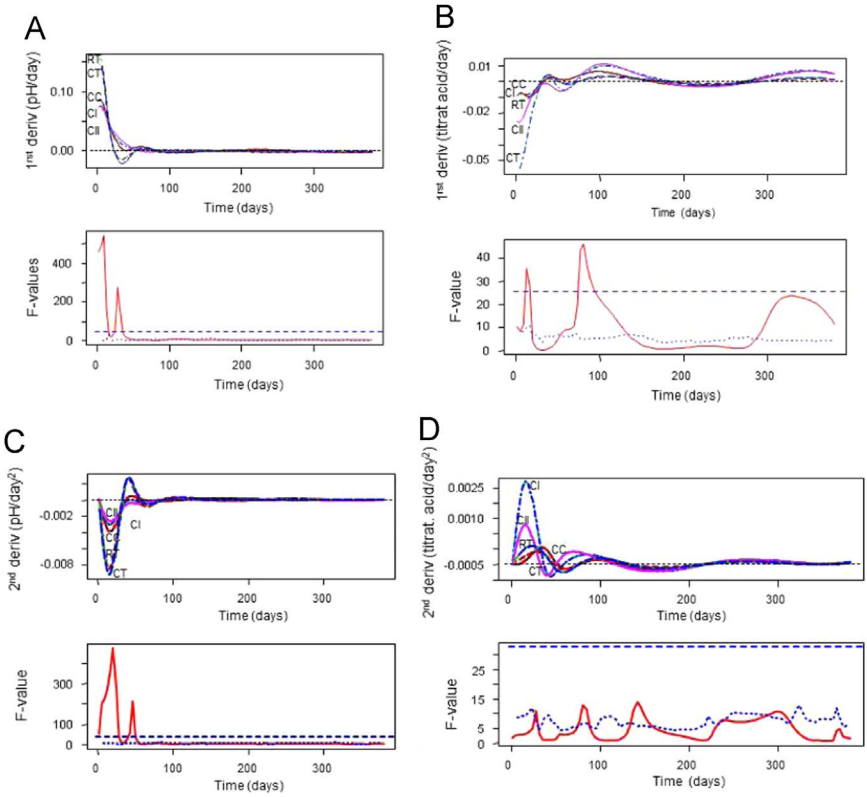

Fig. 6. Functional analysis of variance for first (pH, panel A upper graph; titratable acidity, panel B upper graph) and second derivatives (pH, panel C upper graph; titratable acidity, panel D upper graph), and their respective estimated permutation functional *F*-tests (bottom curves of panels). For the *F*-test, the pointwise *F*-values, together with its maximum (broken lines) and pointwise (dotted line) $p=0.05$ critical values are indicated. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

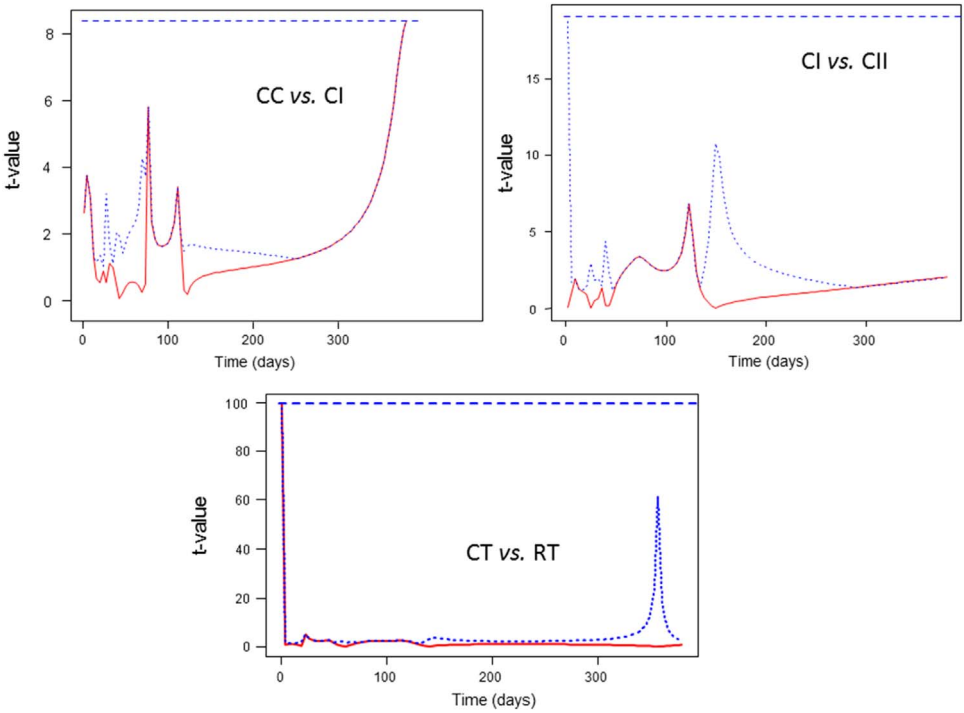

Fig. 7. Functional permutation *t*-test for the comparison of yeast growth curves (CC vs. CI, CI vs. CII, and CT vs. RT). Graphs show the pointwise estimated *t*-test values together with their maxima (broken lines), and pointwise (dotted line) $p=0.05$ critical values. CC, control of acidified cured olives; CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

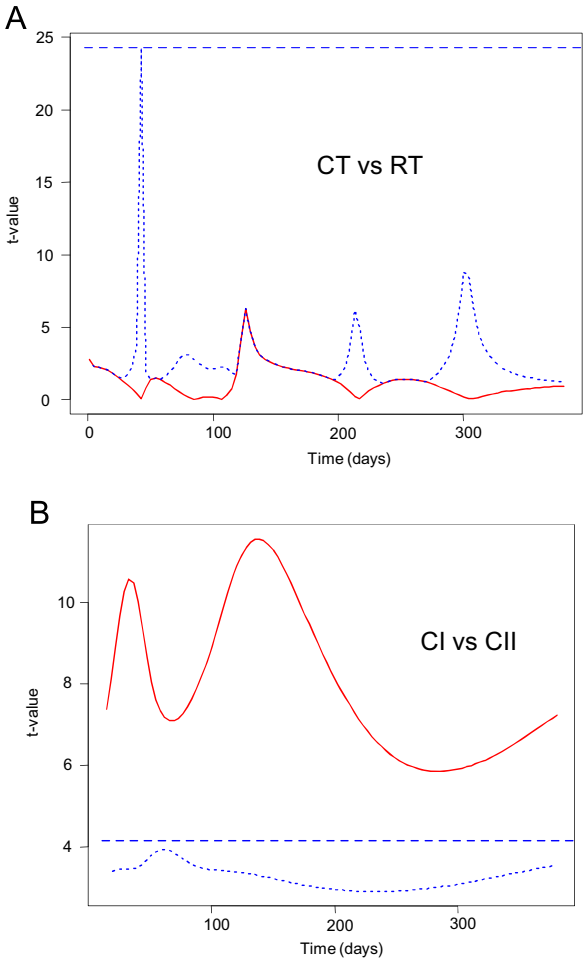

Fig. 8. Permutation functional *t*-test for the comparison of pH changes in CT vs. RT (panel A) and titratable acidity changes in CI vs. CII (panel B). The graphs show the pointwise *F*-values, together with its maximum (broken lines) and pointwise (dotted line) $p=0.05$ critical values. CI, highly acidified cured olives; CII, intermediate acidified cured olives; CT, control of traditional (cracked) olives; RT, traditional olives, cracked after 72 h of exposure to air.

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Transparency document. Supporting information

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dib.2016.09.013.

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3.1.3 CAPÍTULO 3

Assessment of the bacterial community in directly brined Aloreña de Málaga table olive fermentations by metagenetic analysis

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Assessment of the bacterial community in directly brined *Aloreña de Málaga* table olive fermentations by metagenetic analysis

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ABSTRACT

This study uses an "omics" approach to evaluate the bacterial biodiversity changes during fermentation process of natural green cracked *Aloreña de Málaga* table olives, from raw material to fermented fruit. For this purpose, two industries separated by almost 20 km in Guadalhorce Valley (Málaga, Spain) were analysed for obtaining both brines and fruit samples at different moments of fermentation (0, 7, 30 and 120 days). Physicochemical and microbial counts during fermentation showed the typical evolution of this type of processes, apparently dominated by yeasts. However, high-throughput barcoded pyrosequencing analysis of V2–V3 hypervariable region of the bacterial 16S rRNA gene showed at 97% identity the presence of 131 bacterial genera included in 357 operational taxonomic units, not detected by the conventional approach. The bacterial biodiversity was clearly higher in the olives at the moment of reception in the industry and during the first days of fermentation, while decreased considerably as elapse the fermentation process. The presence of Enterobacteriaceae and Lactobacillaceae species was scarce during the four months of study. On the contrary, the most important genus at the end of fermentation was *Celerinatantimonas* in both brine (95.3% of frequency) and fruit (89.4%) samples, while the presence of well-known spoilage microorganisms (*Pseudomonas* and *Propionibacterium*) and halophilic bacteria (*Modestobacter*, *Rhodovibrio*, *Salinibacter*) was also common during the course of fermentation. Among the most important bacterial pathogens related to food, only *Staphylococcus* genus was found at low frequencies (<0.02% of total sequences). Results show the need of this type of studies to enhance our knowledge of the microbiology of table olive fermentations. It is also necessary to determine the role played by these species not previously detected in table olives on the quality and safety of this fermented vegetable.

1. Introduction

Table olives worldwide production nowadays exceeds 2.5 million tons/year, with Spain, Turkey, Egypt, Greek and Italy as the main producer countries (IOC, 2015). This fermented vegetable is prepared with fruits obtained from cultivated *Olea eoropaea* subsp. *europaea* var. *europea* trees and it has an important role in the culture and diet of many Mediterranean countries. Olive fruit cannot be consumed directly from the tree due to its peculiar characteristics (presence of the bitter glucoside compound oleuropein). For this reason, diverse methods have been developed to make them palatable. Although many of them share the general process of brining/salting, fermentation and acidification, they can differ slightly between areas of production. Green Spanish-style, Greek naturally black and ripe Californian styles are the most popular commercial preparations (Garrido-Fernández et al., 1997). However, in the last years, consumers are demanding more traditional and natural homemade seasoned olives. *Aloreña de Málaga* is a traditional green olive preparation from Guadalhorce Valley (Málaga, Spain) with a Protected Designation of Origin (PDO) recognized by the European Union (DOUE, 2012). This olive variety has unique features, related to the production area, which make them quite different from others: its fruits are characterized by an excellent flesh-to-stone ratio, a green–yellow colour, a crispy firmness, and a peculiar mild bitter taste. The manufacturing process is carried out by small and medium enterprises placed in, or very close to, the region of production. Due to its low-to-moderate concentrations of oleuropein, the processing does not include alkaline debittering. Thus, they are produced as directly brined olives and seasoned at the moment of packaging (López-López and Garrido-Fernández, 2006).

In many cases, table olives are produced thorough spontaneous fermentations performed by the indigenous microbiota activity initially present in olive fruit, ingredients, and the environment (fermentation vessels, pipelines, etc.). It is widely accepted that the main microbiota

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with a positive role during table olive fermentations are lactic acid bacteria (mainly *Lactobacillus plantarum* and *Lactobacillus pentosus* species) and yeasts (*Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida boidinii*, among others), opposite to the role played by Enterobacteriaceae, *Clostridium*, and Propionibacteriaceae which are considered undesirable microorganisms (Arroyo-López et al., 2012; Garrido-Fernández et al., 1997; Hurtado et al., 2012).

Diverse molecular methods have been used to study the bacterial community associated to *Aloreña de Málaga* table olives fermentation and packaging. Because the presence of high concentrations of antimicrobial compounds, as occurs in other directly brined table olive specialities, it is assumed that the fermentation process is mainly dominated by yeasts (López-López and Garrido-Fernández, 2006). However, recently Abriouel et al. (2012) uses REP-PCR clustering and further identification of strains by sequencing of *phes* and *rpo* genes for the study of the LAB population associated to this table olive speciality, while Romero-Gil et al. (2016) used sequencing of ribosomal 16S gene and multiplex PCR of *recA* gene for the study of the Enterobacteriaceae and Lactobacillaceae populations, respectively. Unfortunately, the use of methods that rely on the cultivation of microorganism in selective media do not offer a complete profile of the microbial diversity that is present in olive fruit fermentation ecosystem and only a small portion of the true microbial population is detected. For this reason, Abriouel et al. (2011) used a culture-independent approach (PCR-DGGE) for the study of the bacterial biodiversity in *Aloreña de Málaga* fermentations. All these studies were performed exclusively with brines and they did not take into consideration the study of the microbial population adhered to olive surface, which is finally the food intake by consumers.

Metagenetics has become ubiquitous in the field of microbial ecosystem exploration and diverse natural environments (water, air, soil, plants, digestive tract, etc.) have been thoroughly explored by this approach. High-throughput sequencing has also emerged as a new culture-independent tool to quantitatively investigate the biodiversity of microbial communities in foods in order to look at dominant as well as minor microbial populations, gaining at the same time information of the fermentative process and the microbiota of raw materials (Ercolini, 2013; Kergourlay et al., 2015). It has revolutionized the field of food microbial ecology via more accurate identification of microbial taxa without the need for cultivation-dependent methods. In the specific case of table olive fermentations, recently Cocolin et al. (2013) and De Angelis et al. (2015) have used this powerful methodology for the study of the bacterial biodiversity adhered to the surface of diverse Italian olive varieties (*Nocellare etnea* and *Bella di Cerignola*) using the 16S rRNA encoding gene as masker.

The aim of this study was to use a phylogeny metagenetic approach to evaluate the changes in bacterial community through raw material until end of fermentation of PDO *Aloreña de Málaga* table olives, to rationally assess the influence of industry, substrate and time on their population dynamics. Insight into the bacterial life of table olive fermentation will allow us to obtain valuable information of the fermentation process for the design of new strategies to improve the quality and safety of this fermented vegetable.

2. Material and methods

2.1. Sampling of industrial fermentations

Samples were obtained from industrial fermentations of PDO *Aloreña de Málaga* table olives during October 2014 to January 2015. Fruits were harvested at green maturation stage, washed to remove impurities, cracked and directly brined in a 110 g/L NaCl solution in fermentations vessels with 220 L capacity (130 kg fruits). When necessary, fermentation vessels were required with new brine of 120 g/L NaCl and 13 g/L citric acid. Two different industries (labelled as COP and TOL) located at Guadalhorce Valley (Málaga, Spain) were sampled by duplicate. Both industries are separated by a distance of 19.2 km but they produce the same denomination of product (traditional PDO *Aloreña de Málaga* olives). Samples were obtained from fermentation brines (B) and fruits (F) at the time of reception in the factory (fresh fruit, FF) and after 7 (initial stage of fermentation), 30 (minimum time of brining contemplated by PDO *Aloreña de Málaga* normative) and 120 (moment of packaging established by demand) days of fermentation (0, 1 and 4 months, respectively). Table 1 shows the references of the samples analysed in the present study and their characteristics.

2.2. Monitoring of the fermentation process

The analyses of olive brine for pH, NaCl, titratable and combined acidity were carried out using the routine methods described by Garrido-Fernández et al. (1997). Firmness and surface colour of fruits followed methods described elsewhere (Bautista-Gallego et al., 2011), determining the CIE parameters: L^* (lightness), a^* (freshness, negative values indicate green while positive values are related to red tones), and h_{ab} (hue angle). Individual reducing sugars (glucose, fructose, sucrose and mannitol) were determined by HPLC according to the methods developed by Sánchez et al. (2000).

For the counts of the Enterobacteriaceae, yeasts and Lactobacillaceae populations in both brine and fruit samples were spread in selective media according to methods described by Rodríguez-Gómez et al. (2015). Counts were expressed as $\log_{10}$ CFU/mL for brines or $\log_{10}$ CFU/g for olives.

2.3. DNA extraction from olive matrix, preparation of libraries and pyrosequencing

All samples were treated in the same day for DNA extraction from solid (fruit) or liquid (brine) matrixes. In the case of fermentation brine samples, a volume of 50 mL was taken from fermentation vessels and spun at 14,000 rpm for 20 min at 5 °C. Then, the pellet was washed twice in saline solution (0.9% NaCl). In the case of fruit samples, 20 g of pulp (approximately 4–5 pitted olives) was homogenized with 50 mL of saline solution in a stomacher for 2 min and the aqueous phase was spun to get a pellet with same conditions describe above. DNA isolation was done using the PowerFood® Microbial DNA Isolation Kit (MoBio, Carlsbad, Calif.) according to the manufacturer instructions. Purified DNA samples (~10 ng/μL) were stored at −20 °C until use.

A total of 14 different samples by duplicate (Table 1) were used for bacterial community analysis. The 28 DNA samples were submitted to PCR-amplification of the V2-V3 hypervariable region of the bacterial 16S rRNA gene. Three independent 20-μL PCRs were performed for each sample using a two-step PCR protocol with the 16S rRNA gene primers 27F (5′-*AGTTTGATCCTGGCTCAG*-3′) and 357R (5′-*CTGCTGCCTYCCGTA*-3′) linked to universal M13/pUC forward (5′-*GTTGTAAAACGACGGCCAGT*-3′) and M13/pUC reverse (5′-*CACAGGAAACAGCTATGACC*-3′) primers (M13F-*27F* and M13R-*357R*) in an approach similar to that described before (Gholami et al., 2012). Then, second PCR reactions were performed using a 10× dilution of the first PCR product with the fusion forward primer of the Lib-L consisting of the A-adaptor sequence 5′-*CCATCTCATCCCTGCGTGTCTCCGAC*-3′ followed by the 4-base calibration sequence 5′-*TCAG*-3′, a 10-base MID oligonucleotide to differentiate each of the 28 samples and the 20-base M13F/pUC forward oligonucleotide. The reverse fusion primer consists of the Lib-L B-adaptor sequence 5′-*CCTATCCCCTGTGTGCCTTGGCAGTC*-3′ followed by the 4-base calibration sequence, and the 20-base M13/pUC reverse oligonucleotide. HPLC-purified oligonucleotides were synthesized by TIB MOLBIOL (Berlin, Germany). All PCR reactions were run in a T100TM Thermal Cycler (Bio-rad, Madrid Spain) using the FastStart High Fidelity Polymerase (Roche Diagnostics GmBH, Mannheim, Germany) and conditions recommended by the manufacturer for pyrosequencing analysis for each type of amplicons. The PCR products were purified twice with AgencourtH AMPureH XP PCR purification system (Agencourt

Table 1
Number of sequences and OTUs assigned (after removing chloroplast), observed diversity and estimated sample coverage for 16S (bacteria) amplicons for the different type of samples after grouping duplicated experiments.

Sample	Matrix	Industry	Time	Number of reads	Number of OTUs	Good's coverage	PD whole tree[a]	Chao1[a]	Richness[a]
FF-COP	Fresh fruit	COP	0 month (0 days)	817	85	96.94	5.40	79.20	64.40
F-COP-0	Fruit	COP	0 month (7 days)	723	152	88.38	8.36	230.23	101.10
F-COP-1	Fruit	COP	1 month (30 days)	2398	69	98.37	2.63	49.76	25.10
F-COP-4	Fruit	COP	4 months (120 days)	1051	75	96.29	4.20	102.76	35.90
B-COP-0	Brine	COP	0 month (7 days)	355	65	90.70	4.57	111.39	64.40
B-COP-1	Brine	COP	1 month (30 days)	510	145	84.90	9.19	221.03	117.20
B-COP-4	Brine	COP	4 months (120 days)	4387	69	99.07	1.57	23.78	11.70
FF-TOL	Fresh fruit	TOL	0 month (0 days)	1404	137	96.94	7.14	135.73	83.80
F-TOL-0	Fruit	TOL	0 month (7 days)	410	78	90.49	5.74	126.30	72.50
F-TOL-1	Fruit	TOL	1 month (30 days)	1956	27	99.23	1.27	25.48	10.60
F-TOL-4	Fruit	TOL	4 months (120 days)	2312	36	98.96	1.28	31.95	11.10
B-TOL-0	Brine	TOL	0 month (7 days)	4345	89	98.76	1.93	56.23	16.00
B-TOL-1	Brine	TOL	1 month (30 days)	7700	61	99.51	1.35	17.96	8.90
B-TOL-4	Brine	TOL	4 months (120 days)	4433	47	99.35	1.59	17.13	9.10

[a] Values were estimated after rarefaction to 350 sequences.

Bioscience Co., Beverly, MA, USA) and quantified using the QuantiT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA) and a fluorometer (BioTek Instruments, Winooski, VT, USA). Subsequently, all samples from each run were pooled in equimolar concentrations and purified again twice with AgencourtH AMPureH XP PCR. Pools of the 28 samples were diluted to obtain a total of 1×10^5 copies/μL and two independent emulsion PCRs were performed with the Lib-L kit (454 Life Sciences) according to manufacturer's instructions for short (16S) reads. DNA positive beads were enriched, counted on the GS Junior Bead Counter, and loaded onto a picotiter plate for pyrosequencing on the 454 Life Sciences (Roche) Junior platform according to the standard platform protocols for short (16S) sequencing runs. Two independent runs were obtained for each 16S sequences. Additionally, other 18 samples were run in a third run to increase the number of sequences from some samples and test the reproducibility of results.

2.4. Statistical analysis of pyrosequencing reads

Samples were processed and analysed following the procedure described by Caporaso et al. (2010) using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (version v1.9.1. http://qiime.sourceforge.net/) using default parameters unless otherwise noted. Sequences were first screened for quality using the following parameters: minimum quality score of 25, minimum sequence length of 200 bp, maximum length of 600 bp (16S), and no ambiguous bases in the entire sequence or mismatches in the primer sequence. Any sequences not meeting these parameters were excluded from downstream analyses. Sequences were then sorted by barcode into their respective samples and the barcode and primer sequences were removed. Chimeras were removed and operational taxonomic units (OTUs) were clustered de novo (pick_de_novo_otus.py script) using USEARCH at 97% identity (Edgar, 2010). Sequences are available at the Sequence Read Archive of Genbank under BioProject ID PRJNA315418. Taxonomy was assigned to the OTUs against the SILVA 108 database preclustered at 97% identity (McDonald et al., 2012) (http://qiime.org/home_static/dataFiles.html). A phylogenetic tree was constructed using the FastTree 2.1.3 with default parameters (Price et al., 2010) for use in phylogenetic diversity calculations. Singleton OTUs were filtered out of the entire dataset to reduce the noise caused by PCR or sequencing error.

Data from the replicated fermentations vessels were combined before statistical analysis. Data analyses were performed at the genus taxonomy level. Differences between bacterial communities were calculated in QIIME using rarefaction curves of alpha-diversity indexes including estimates of community richness (such as the Chao1 estimator, richness or the observed number of OTUs present in each sample, good's coverage, and phylogenetic diversity (PD) or the amount of phylogenetic branch length observed in each sample). These alpha-diversity indexes were chosen to estimate the total diversity in the different microbial communities for each substrate and industry and each sampling time (Lozupone and Knight, 2008). Rarefaction analysis was performed using rarefied OTU tables (rarefied to 350 sequences); the lowest number of reads obtained for any of the 28 DNA samples analysed to control for differing depths of sequencing across the samples, 100 replications, and cut-offs of 97% sequence similarity. Beta-diversity UniFrac distance matrices were built for 16S sequences only after subsampling all samples to an even depth of 350 sequences per sample. UniFrac distances were based on the PD beta-diversity measures to evaluate the extent to which microbial communities changed over time, or between substrates or industries (Lozupone and Knight, 2008). Taxonomic abundances within each identified Phylum to genus level were visualized using Krona hierarchical data browser (Ondov et al., 2011). Principal coordinates analysis (PCoA) was also performed on the UniFrac distance matrices to show the differences between the sample types, and visualized using the KiNG graphics program (http://kinemage.biochem.duke.edu/software/king.php). Statistical significance of differences in alpha- and beta-diversity were performed with QIIME using a nonparametric two sample *t*-test with 999 Monte Carlo permutations on number of observations, Chao1 and PD and nonparametric ANOSIM tests on unweighted UniFrac (16S).

3. Results

3.1. Monitoring of the fermentation process

The fermentation process of traditional *Aloreña de Málaga* table olives was followed during four months by routine physicochemical and microbiological analyses. Table 2 shows the evolution of the main physicochemical characteristics assayed in both brines (pH, salt, titratable and combined acidity, sugar concentration) and fruits (colour and texture). In general, the evolution of physicochemical parameters was similar in both industries, except salt concentration which was slight higher in COP industry at the onset of fermentation (80.1 COP vs 66.8 g/L TOL). Then, the salt concentration increased up to practically 95 g/L at the end of fermentation process in both factories by the addition of new brine. The profile of pH in brines was kept practically constant during all fermentation process, ranging from 4.31 to 4.53. The combined acidity was also kept constant during all time approximately at 0.10 Eq/L, while titratable acidity slight increased from approximately 0.40% to 0.60%. The initial total sugar concentration in brine was around 21 g/L, principally composed by glucose. Glucose, fructose and sucrose were consumed by microorganisms during fermentation process. However, the content in mannitol remained unchanged during four months, with a higher content in COP industry (Table 2). Regarding physicochemical characteristics of the fruits, texture was not affected during

Table 2
Physicochemical evolution of the brines and fruits during fermentation process in the different industries. Standard deviation from duplicate experiments in parentheses.

Time (days)	pH		Salt (g/L)		Titratable acidity (%)		Combined acidity (Eq/L)	
	COP	TOL	COP	TOL	COP	TOL	COP	TOL
7	4.47 (0.02)	4.53 (0.03)	80.1 (0.80)	66.8 (2.10)	0.39 (0.01)	0.42 (0.01)	0.09 (0.00)	0.11 (0.00)
30	4.48 (0.03)	4.31 (0.12)	81.6 (1.11)	76.0 (0.50)	0.59 (0.04)	0.57 (0.04)	0.11 (0.01)	0.10 (0.00)
120	4.48 (0.03)	4.45 (0.01)	92.4 (0.50)	95.5 (0.40)	0.54 (0.07)	0.62 (0.05)	0.09 (0.00)	0.08 (0.00)
Time (days)	Glucose (g/L)		Fructose (g/L)		Sucrose (g/L)		Mannitol (g/L)	
	COP	TOL	COP	TOL	COP	TOL	COP	TOL
7	12.12 (0.69)	14.49 (1.39)	2.55 (1.05)	3.05 (0.58)	1.29 (0.01)	1.22 (0.17)	4.61 (0.55)	2.78 (0.00)
30	6.54 (0.40)	4.15 (0.03)	1.75 (0.00)	0.89 (0.01)	0.79 (0.18)	0.37 (0.04)	4.75 (0.31)	2.86 (0.08)
120	2.85 (0.32)	3.30 (0.22)	0.89 (0.00)	0.99 (0.02)	0.10 (0.02)	0.12 (0.00)	4.16 (0.52)	2.68 (0.11)
Time (days)	Texture (KN/100 g)		Colour a^*		Colour h_{ab}		Colour L^*	
	COP	TOL	COP	TOL	COP	TOL	COP	TOL
7	6.27 (0.28)	6.36 (0.02)	−4.97 (1.03)	0.10 (1.46)	74.07 (1.89)	66.45 (2.31)	61.70 (2.16)	58.96 (2.38)
30	6.34 (0.34)	6.82 (0.35)	5.24 (0.63)	4.36 (0.08)	82.43 (1.43)	83.24 (0.32)	55.20 (1.99)	53.96 (1.35)
120	6.76 (0.67)	6.76 (0.19)	6.24 (0.14)	6.44 (0.71)	80.62 (0.50)	79.52 (0.86)	54.50 (0.50)	53.16 (0.85)

four months of fermentation, ranging from 6.27 to 6.82 KN/100 g, while colour of fruits was characterized by a loss of green appearance (a^* parameter increased from negative to positive values) and luminosity (L^* parameter decreased from initial 60 to approximately 54 through fermentation process).

As regards microbial counts, Enterobacteriaceae and Lactobacillaceae were below limit of detection (<1.3 $\log_{10}$ CFU/mL) during all fermentation process, in both brines and fruits. On the contrary, yeasts increased during time studied, with population levels higher in TOL than in COP industry for much of the time of fermentation. After four months of fermentation, this fungal group reached practically the same population in both industries, with ~5.0 $\log_{10}$ CFU/mL in brines, and ~4.5 $\log_{10}$ CFU/g in fruits.

Thus, the main physicochemical and microbiological changes which occurred during fermentation were related with sugar consumption (mainly glucose), a slight salt and titratable acidity increase, loss of green colour and luminosity of fruits (darkening), and yeast dominance. All these changes were very similar in both factories and can be considered as usual during fermentation of this speciality of natural, cracked, green olives. The flavour and aroma of fermented olives were also tested by a training panel, not detecting the presence of abnormal taste or smells and resulting in the typical product (data not shown). Hence, the samples obtained for pyrosequencing analysis can been considered as representative of this type of process for both industries.

3.2. Phylogenetic analysis of the bacterial community

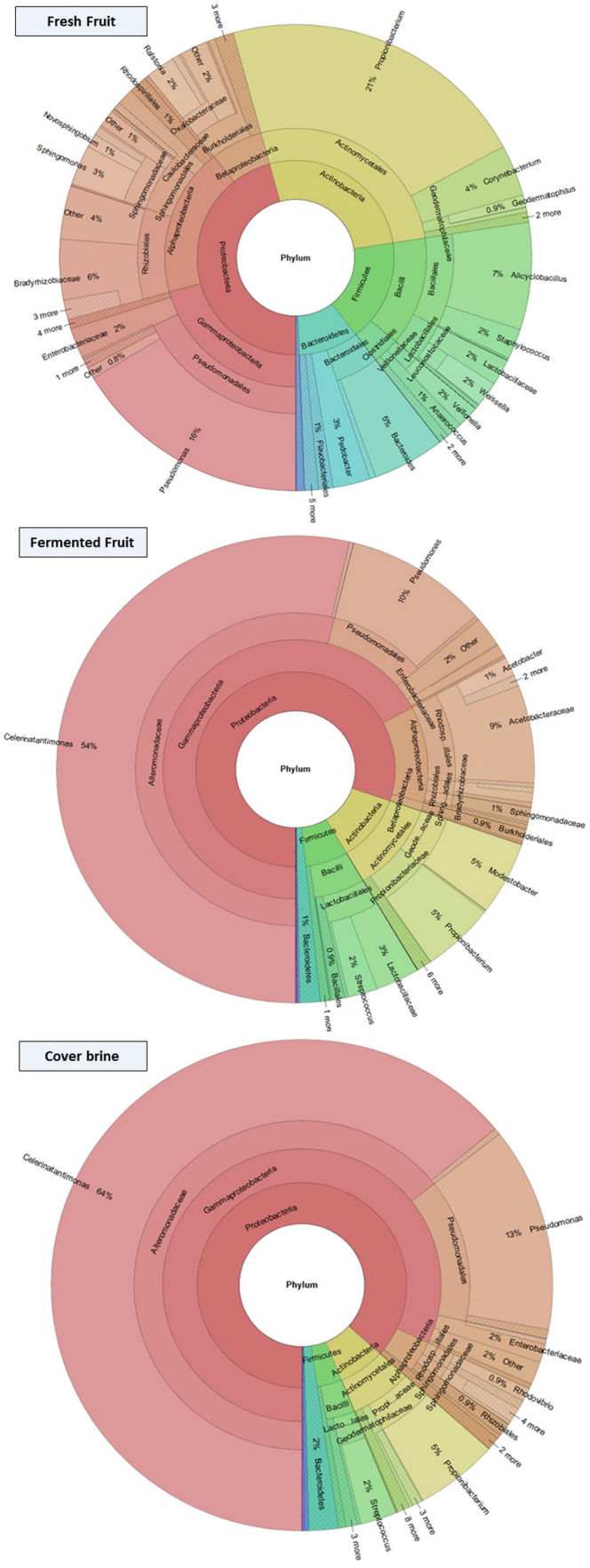
The pyrosequencing of the 16S-PCR products generated a total of 337,114 raw sequences for the 28 olive samples. After screening our data for poor quality sequences, we recovered 274,141 high quality sequences with an average of 9791 sequences per sample. From those, we obtained a total of 254,147 sequences that could be assigned into OTUs with a mean of 5906 classifiable sequences per sample. After removing chloroplasts and taxonomically unassigned 16S sequences, a total of 32,801 sequences were finally used for metagenetic analysis making a mean of 1171 sequences per sample.

Across all taxa, 131 bacterial genera and 357 OTUs, with an average of 81 observed OTUs per sample, were identified (see Table 1). The bacterial phylogenetic characterization of all samples showed big differences between fresh fruits, fermented fruit and brine samples, and covered four main bacterial phyla including Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes (Fig. 1). However, whereas for fresh fruits samples, Alphaproteobacteria, Gammaproteobacteria, Bacilli and Actinomycetales classes were present in similar proportions (13.6 to 27.0%), for fermented fruit and brine samples this proportions where completely shifted to a significantly higher proportion of Gammaproteobacteria of the family Alteromonadaceae (66.9% for fermented fruit and 81.8% for brines; being significantly higher for brine samples). Also, the proportion of Alphaproteobacteria, Actinobacteria, Bacilli and Betaproteobacteria were significantly higher for fermented fruits than for brines. From the total of 131 genera identified, only 21 genera represented >90% of the total sequences, which ranged between 0.5% (*Pedobacter*) to 50.6% (*Celerinatantimonas*). Globally, for fermented fruits the genera that accounted for >80% of the sequences were: *Celerinatantimonas* (53.5%), *Pseudomonas* (9.7%), unknown Acetobacteraceae (6.8%), *Modestobacter* (5.1%), *Propionibacterium* (5.0%), and an unknown Lactobacillaceae (3.0%), whereas for brines were *Celerinatantimonas* (63.9%), *Pseudomonas* (13.4%), and *Propionibacterium* (5.4%) (Fig. 1).

A change of bacterial genera was observed during fermentation process. Thus, the fermented fruit samples at 7th day of fermentation showed high levels of *Pseudomonas* sp. (27.1%), *Modestobacter* sp. (15.1%), *Propionibacterium* (13.5%, mainly *P. acnes*), and an unidentified Lactobacillaceae (8.8%), and very low numbers of *Celerinatantimonas* (1.8%) or members of Acetobacteraceae (<1%), whereas at 30th day of fermentation most bacteria belonged to *Celerinatantimonas diazotrophica* (69.4%) and unidentified Acetobacteraceae (19.7%), or *Acetobacter* sp. (4%) and a Gammaproteobacteria (2.8%) with very low proportion of *Pseudomonas* sp. and *Propionibacterium* (<0.2%) or undetectable (*Modestobacter* sp., and Lactobacillaceae). Finally, at 120th day of fermentation, most bacteria belonged to *C. diazotrophica* (89.4%) and Gammaproteobacteria (3.2%) with the remaining genera being present at very low proportions or not being detectable (Fig. 2). On the other hand, samples from brines at 7th day of fermentation were dominated by *C. diazotrophica* (46.6%), *Pseudomonas* sp. (34.7%), *Rhodovibrio* sp. (2.3%) and Enterobacteriaceae (2.1%), whereas at 30th day of fermentation most bacteria from brines samples belonged to *C. diazotrophica* (49.7%), *P. acnes* (14.2%) and *Streptococcus* sp. (7.0%), and at 120th day of fermentation most bacteria were represented by *C. diazotrophica* (95.3%) (Fig. 2). Tables S1 and S2 in Supplementary material shows the relative abundance of the most representative OTUs, at genera and family level, in all samples analysed.

3.3. Biodiversity of the bacterial community

The Venn diagrams show that a total of 63 OTUs (48%) were shared among the three substrates (fresh fruits, brines and fermented fruits), with fermented fruit and brine samples sharing the highest number of OTUs (113 OTUs; 86%) and fresh fruit and brine the lowest (69 OTUs; 53%), and a few or none OTUs being unique (non-shared by any other sample) for each sample type (Fig. 3A). Table S3 in Supplementary material shows the OTUs assigned at genera level shared among the three types of substrates. The number of unique and shared bacterial OTUs



changed with the type of substrate and during the fermentation process. Thus, 15 bacterial OTUs (12%) were shared by all fruits (including fresh fruits) through fermentation process, but the highest number of OTUs was shared between fresh fruits and fruits at 7th day after starting the fermentation (F-0). The number of unique bacterial OTUs diminished during fermentation time from F-0 to F-4 (Fig. 3B). Table S4 in Supplementary material shows the OTUs assigned at genera level shared among the fruits in all sampling time. In brine samples, a total of 28 bacterial OTUs (23%) were shared among all sample types with the brines samples at 30th day of fermentation (B-1) showing the highest number of unique OTUs (Fig. 3C). Table S5 in Supplementary material shows the OTUs assigned at genera level shared among the brine samples in the different sampling time. The bacterial genera *Celerinatantimonas*, *Pseudomonas*, *Propionibacterium*, *Salinibacter*, *Staphylococcus*, *Rhodovibrio*, *Streptococcus*, and *Alicyclobacillus* were shared among substrates, fruits and brines in the different sampling times (see Tables S3–S5 in Supplementary material).

The bacterial community was also analysed using rarefaction curves and richness estimator (Chao1 index). The Chao1 index varied from 17.1 (brine sample obtained from TOL industry after 4 months of fermentation) to 230.2 (fruit sample obtained from COP industry at the onset of fermentation) (Table 1). Overall, despite the diversity of sequencing depth between samples, the rarefaction analysis indicated that the number of bacterial reads above 350 per sample was satisfactory to obtain a good coverage. Thus, there was a satisfactory coverage of the bacterial diversity for all the samples analysed with Good's coverage values above 90% with the exception of two treatments (see Table 1). This result was also confirmed by the analysis of rarefaction curves (Fig. 4). When analysing alpha-diversity rarefaction curves for bacteria, we found differences among fermented fruit and brines samples, industries and during the fermentation process, with similar pattern for all alpha-diversity indexes (Fig. 4; only Richness data are shown). For both factories, alpha-diversity was higher for fresh fruits and after 7th days of fermentation; then significantly decreased at 30th and 120th days of fermentation. For brine samples, there were significant differences among industries with samples from TOL industry showing low alpha-diversity values, whereas for COP industry there was a trend to increase alpha-diversity values at 30th day of fermentation and then a dramatically decreased occurred at 120th day of fermentation reaching value similar to those obtained for COP industry at same period of fermentation.

Unweighted UniFrac analysis based in principal coordinates analysis of 16S sequences segregated olive fruits samples unprocessed (FF) and those at the beginning of the fermentation process (F-0) irrespective of the industry along PC1 that explained >79% of total variance. These samples were also closer to brines samples from COP industry at 7th and 30th days of fermentation due to their higher alpha and beta-diversity values. On the contrary, all fermented fruit and brines samples for both industries tended to group together at 30th and after 120th days of fermentation with low distance values among them indicating a closer similarity in their bacterial communities (similar PC1 and PC2 values) pointing out that the changes occurring during the fermentation process (time) were the main drivers of microbial community composition irrespective of the substrate or industry (Fig. 5). However, ANOSIM test indicated that there were not statistical significant differences ($p > 0.05$) among the unweighted UniFrac distances when comparing samples among the different categories (i.e., industry, substrate, or fermentation time).

4. Discussion

Metagenetic analysis has been used to investigate the changes in bacterial communities in diverse vegetables in brines such as asparagus,

Fig. 1. Taxonomic abundances (%) from phylum to genus level in the fresh fruits at the moment of reception in the industry, fermented fruit and brine samples. The different industries and sampling times were considered together for elaboration of the graphs.

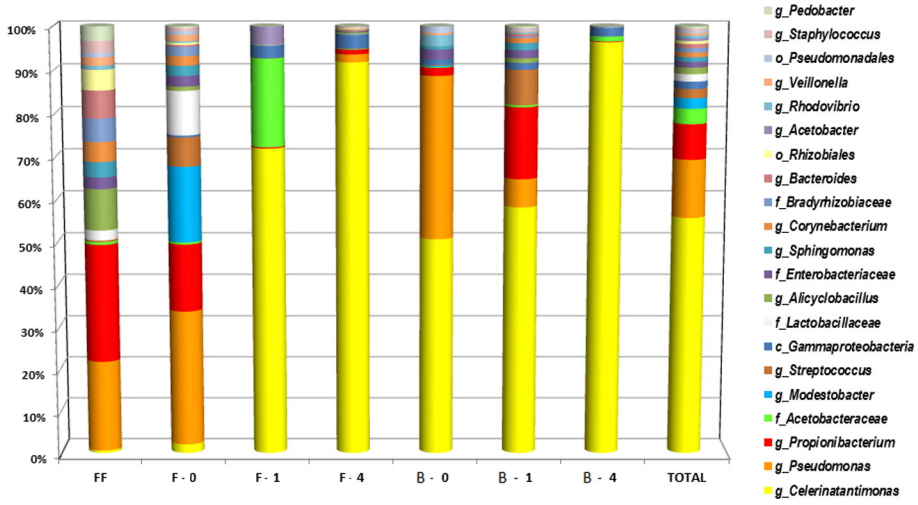


Fig. 2. Relative abundance (%) of genera or family obtained by pyrosequencing analysis through fermentation process. FF, F, and B stands for fresh fruits, fermented fruits and fermentation brines, respectively, while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation, respectively).

cucumbers, kimchi, and table olives. This way, the bacterial population of green asparagus was composed mainly by Proteobacteria (mainly *Pantoea* and *Pseudomonas* genera), followed by Firmicutes (mainly *Lactococcus* and *Enterococcus*) (del Árbol et al., 2016). Bacterial community of kimchi was represented mainly by the genera *Leuconostoc* and *Lactobacillus*, but also of *Weissella*, *Pantoea* and *Pseudomonas* (Jeong et al., 2013). Medina et al. (2016) reported recently the presence of *Acetobacter*, *Gluconobacter* and *Lactobacillus* as the majority genera during fermentation of cucumbers. In the specific case of table olives, Cocolin et al. (2013) used pyrosequencing analysis for the study of the bacterial ecology during fermentation of directly brined *Nocellare etnea* olives. They found also a change of the bacterial population through fermentation process. This way, the surface and brines of the olives at the onset of fermentation was characterized by a high level of halophilic bacteria, mainly *Chromohalobacter*, *Halomonas*, and *Marinilactibacillus* genera, while after 3 months of fermentation the structure of the population changed dramatically, especially in olive surface with *Lactobacillus* as the main bacterial population present. These authors also reported the presence of *Pseudomonas* and *Propionibacterium* among the DNA samples, but at low frequencies (<1.5% in the highest case). De Angelis et al. (2015), using also pyrosequencing analysis for the study of bacterial changes trough fermentation of not lye-treated *Bella di Cerignola* olives, found that the main genus present in the fresh olives and the onset of fermentation were *Hafnia* and *Methylobacterium*, whilst at the end of fermentation were *Lactobacillus* and *Propionibacterium*.

In this study, *Pseudomonas*, *Modestobacter*, *Acetobacter* and *Propionibacterium* (*P. acnes*) were the genera that accounted for the majority of sequences in both fruit and brine samples at the onset of fermentation, whilst at the end of fermentation most bacteria belonged to *Celerinantimonas* genera (*C. diazotrophica*). The presence of Enterobacteriaceae and Lactobacillaceae during all fermentation process (4 months) was scarce, in contrast with studies described before in table olives and other fermented vegetables. This way, Lactobacillaceae only represented globally the 2% of total sequences in the fresh fruit and 3% in fermented fruits, while the frequency of Enterobacteriaceae was approximately 2% in the different type of substrates. The presence of both bacterial taxa in the fermentation of table olives and other vegetables is habitual, with a well-known negative role during fermentation for Enterobacteriaceae, and positive for Lactobacillaceae (Garrido-Fernández et al., 1997). Among the most important bacterial pathogens related to foods (*Listeria*, *Clostridium*, *Escherichia*, *Salmonella*, etc.), only *Staphylococcus* genus was found at low frequencies (<0.02% of total sequences) in the present study. This result has a special relevance regarding safety issues. In general, the presence of food-borne pathogen in table olives is scarce, as it was also confirmed by pyrosequencing analysis by Cocolin et al. (2013) and De Angelis et al. (2015), who only found a low abundance of the genera *Escherichia*, *Staphylococcus*, *Clostridium* and *Listeria* during fermentation process of diverse *Italian* olive varieties.

The bacterial biodiversity in the different samples was affected by the type of industry, with in general lower biodiversity indexes in TOL factory than in COP, and by the time of fermentation, with this later factor being the major driver of both alpha- and beta-diversity changes. Thus, the chao1 and richness indexes had lower values at the end of fermentation compared to initial points, indicating that both the total number of OTUs (richness) and the number of rare or less frequent

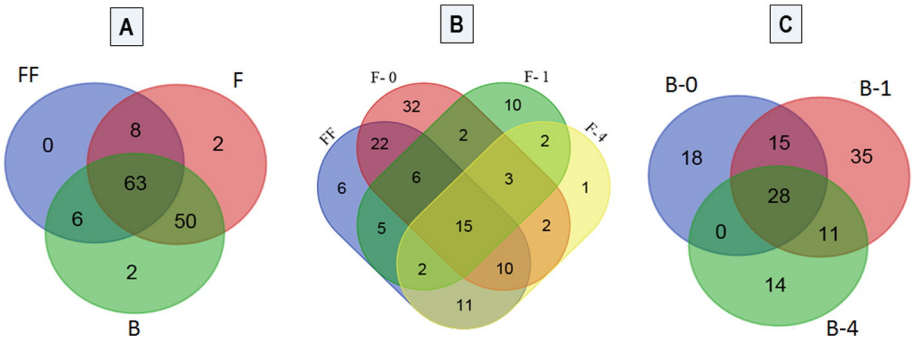


Fig. 3. Venn diagrams showing the number of unique and shared OTUs among substrates (A), sampling times in fruits (B) and sampling times in cover brines (C). FF, F, and B stands for fresh fruits, fermented fruits and cover brines, respectively, while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation, respectively).

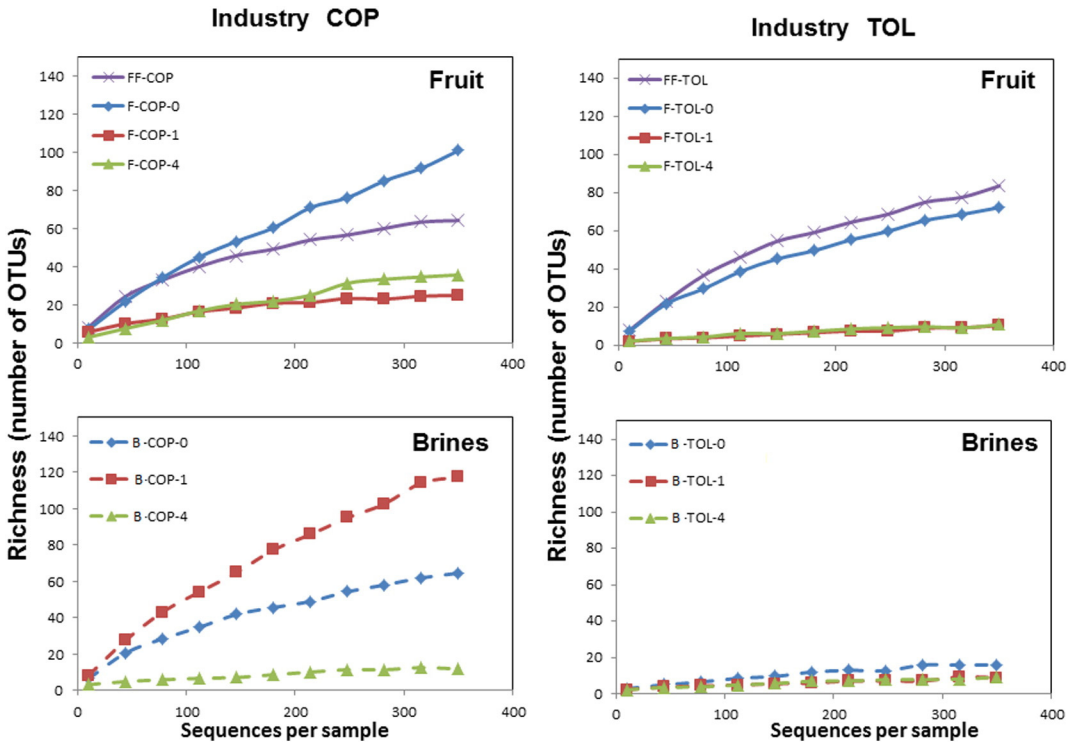


Fig. 4. Rarefaction curves for the different industries and substrates. FF, F, and B stands for fresh fruits, fermented fruits and cover brines, respectively, while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation, respectively).

OTUs (Chao1) tended to decrease during the fermentation process, and the beta-diversity UniFrac distances tended to decrease in the same direction. This is indicative that during course of fermentation, only determined species (mainly *C. diazotrophica*) were adapted to the fermentation process. This contrast with data obtained by Cocolin et al. (2013) and De Angelis et al. (2015), who noticed similar biodiversity indexes between the initial and end points of olive fermentations. *C. diazotrophica* has been recently described by Cramer et al. (2011) as a facultative anaerobic, nitrogen-fixing, short, motile, polar monotrichous rods bacterium, belonging to the class Gammaproteobacteria and order Alteromonadales. The type strain of this species was originally isolated from the roots of estuarine grasses *Spartina alterniflora* and *Juncus roemerianus* (Cramer et al., 2011). This species growth between pH 3.5 to 8.0 and it has halotolerance above 80 g/L NaCl, so presumably can support the usual pH and salt conditions found in olive fermentations (Garrido-Fernández et al., 1997). *C. diazotrophica* is metabolically versatile, with ability to ferment glucose to acid products and utilization of a wide variety of carbohydrates (many of them present in olive fruits) and carboxylic acids, as performed by many Lactobacillaceae species (Cramer et al., 2011). Recently, this species was detected among the bacterial community present at the final stage of Sichuan fermentation, a typical representative of Chinese traditional food where different vegetables (cabbage, radish, leaf mustard, bamboo, shoot, tender ginger, and chili) are immersed in salt brine (60–80 g/L) at pH 4.5 (Li et al., 2014), similar to conditions found in this work. Curiously, other genus never detected before in olive samples, *Modestobacter*, has been also isolated from the roots of halophyte plants (*Salicornia europaea*). This genus is classified into the family Geodermatophilaceae and has a considerable tolerance to salt (Qin et al., 2013). We hypothesize that the presence in *Aloreña de Málaga* fermentations of both halophyte genera,

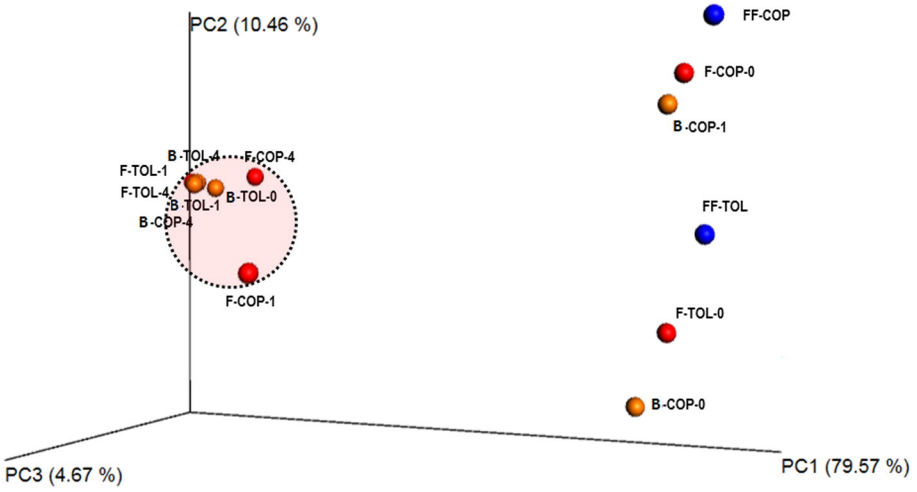


Fig. 5. Unweighted UniFrac analysis based in principal coordinates analysis of 16S sequences obtained from different samples. FF, F, and B stands for fresh fruits, fermented fruits and brines, respectively, TOL and COP stands for different industries, while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation, respectively).

related with marine environment, could be due to the use of sea salt in olive fermentations, which is usually added to prepare the cover brines. This hypothesis is reinforced by the presence also in *Aloreña* samples, although at lower abundance (<0.03%), of the genera *Rhodovibrio* and *Salinibacter*, two halophilic bacteria related with salterns (Johannes, 2005). Their influence (positive or negative) on the fermentative process must be elucidated in further studies.

On the contrary, other microorganisms detected in *Aloreña de Málaga* samples by pyrosequencing analysis are clearly considered undesirable in the fermentation process. The presence of *Pseudomonas* genera during fermentation of *Aloreña de Málaga* table olives was previously described by Abriouel et al. (2011) using a culture-independent approach based in PCR-DGGE analysis. This ubiquitous microorganism has been also detected previously on the surface of unfermented black olives, fermentation of naturally black olives and directly brined *Nocellare etnea* olives (Cocolin et al., 2013; Ercolini et al., 2006; Nychas et al., 2002). *Pseudomonas* are usually associated to fresh foods. Due to its high metabolic potential, diverse species of this group can produce alteration of foods. The development of proteolytic pathways in table olives, followed by decarboxylation and deamination of the resulting amino acids by heterofermentative lactobacilli could cause an unusual type of spoilage characterized by a decrease in the acidity of brines and swelling (Harmon et al., 1987), and could also lead to biogenic amine formation. *Propionibacterium* and *Acetobacter* genera were also detected during the course of this research. *Acetobacter* spp. have been related with spoilage of cucumber fermentations, producing the consumption of lactic acid and consequent formation of acetic acid in aerobic conditions (Medina et al., 2016). An undesired secondary fermentation or spoilage may be initiated by propionic acid bacteria, in particular *Propionibacterium* spp., which is a well-known species in table olive fermentations (Garrido-Fernández et al., 1997). This genera metabolizes sugars or the lactic acid form during the primary fermentation, to produce propionic acid, acetic acid and CO_2, inducing an increase in pH and volatile acidity (Gonzalez-Cancho et al., 1980). These conditions also encourage the development of *Clostridium* species, which together with *Propionibacterium* can promote *zapatería* spoilage, giving abnormal odours and tastes in table olives (Garrido-Fernández et al., 1997). Control of pH and salt concentration in brine would prevent the growth of these spoilage microorganisms and their off-odours fermentations, especially when temperatures are warmer during the summer months (Gonzalez-Cancho et al., 1970).

5. Conclusions

New bacterial species have been detected for the first time in natural green olive fermentations by the use of high-throughput pyrosequencing analysis. Spread of samples on specific selective media only provides partial and very limited information of the microbiology of table olive fermentations. Thus, results show the need of this type of work to improve our knowledge of the microbiology of table olive fermentations. Further studies are also necessary to determine the influence of these new microbial species on the quality and safety of table olives. Apparently, the presence of spoilt microorganisms did not alter the physicchemical characteristics of fermented olives, whilst the absence of pathogens genera reinforces the safety issues of this fermented vegetable.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2016.07.014.

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Table S1. Relative abundance (%) of the more representative OTUs at genus level in the different type of samples analysed.

Taxonomy	FF.TOL	F.TOL.0	F.TOL.1	F.TOL.4	B.TOL.0	B.TOL.1	B.TOL.4	FF.COP	F.COP.0	F.COP.1	F.COP.4	B.COP.0	B.COP.1	B.COP.4
g__Celerinatantimonas	0.4%	1.5%	94.8%	95.2%	92.9%	96.3%	96.3%	0.5%	2.1%	44.1%	83.5%	0.3%	3.1%	94.3%
g__Alicyclobacillus	2.8%	1.0%	0.1%	-	-	-	-	12.0%	0.7%	0.1%	0.8%	-	1.8%	-
g__Pseudomonas	32.4%	47.8%	0.1%	-	2.4%	-	-	0.2%	6.4%	0.1%	3.5%	67.0%	11.2%	-
g__Propionibacterium	18.2%	6.3%	0.1%	-	0.1%	-	0.2%	24.4%	20.9%	0.4%	2.1%	3.4%	28.4%	0.1%
g__Rhodovibrio	1.4%	-	-	-	0.1%	-	-	-	0.6%	-	0.1%	4.5%	-	0.1%
g__Streptococcus	0.4%	2.0%	0.1%	-	-	-	-	0.2%	9.7%	0.1%	0.2%	0.3%	13.9%	-
g__Staphylococcus	-	0.5%	0.1%	-	-	-	-	3.8%	0.7%	-	0.3%	-	0.6%	0.1%
g__Corynebacterium	3.1%	2.2%	-	-	-	-	-	4.0%	1.5%	-	0.5%	-	2.0%	-
g__Bacteroides	3.8%	-	-	-	-	-	-	6.1%	0.7%	-	-	-	1.0%	-
g__Pedobacter	0.2%	-	-	-	-	-	-	4.9%	0.7%	-	-	-	0.6%	-
g__Modestobacter	-	-	-	-	-	-	-	-	30.2%	-	0.1%	0.6%	-	-
g__Acetobacter	-	-	-	-	-	-	-	-	-	7.9%	-	-	-	-
g__Gluconobacter	-	-	-	-	-	-	-	-	-	3.3%	-	-	-	-
Others	37.3%	38.8%	4.9%	4.6%	4.4%	3.5%	3.5%	43.8%	26.0%	44.0%	8.9%	23.9%	37.5%	5.3%

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Table S2. Relative abundance (%) of the more representative OTUs at family level in the different type of samples analysed.

Taxonomy	FF.TOL	F.TOL.0	F.TOL.1	F.TOL.4	B.TOL.0	B.TOL.1	B.TOL.4	FF.COP	F.COP.0	F.COP.1	F.COP.4	B.COP.0	B.COP.1	B.COP.4
f__Alteromonadaceae	0.4%	1.5%	95.1%	95.6%	93.4%	96.8%	96.6%	0.5%	2.9%	44.2%	83.5%	0.3%	4.1%	94.5%
f__Alicyclobacillaceae	2.8%	1.0%	0.1%	-	-	-	-	12.0%	0.7%	0.1%	0.8%	-	1.8%	-
f__Pseudomonadaceae	32.4%	47.8%	0.1%	-	2.4%	-	-	0.2%	6.4%	0.1%	3.5%	67.0%	11.2%	-
f__Propionibacteriaceae	18.2%	6.1%	0.1%	-	0.1%	-	0.2%	24.4%	21.2%	0.4%	2.1%	3.4%	28.8%	0.1%
f__Rhodospirillaceae	1.6%	0.7%	-	-	0.2%	-	-	-	0.6%	-	0.5%	4.5%	0.6%	0.1%
f__Streptococcaceae	0.4%	2.0%	0.1%	-	-	-	-	0.2%	9.7%	0.1%	0.2%	0.3%	13.9%	-
f__Staphylococcaceae	0.4%	0.5%	0.1%	-	-	-	-	3.8%	0.7%	-	0.3%	-	0.6%	0.1%
f__Corynebacteriaceae	3.1%	2.2%	-	-	-	-	-	4.0%	1.5%	-	0.5%	-	2.0%	-
f__Bacteroidaceae	3.8%	-	-	-	-	-	-	6.1%	0.7%	-	0.1%	-	1.0%	-
f__Sphingobacteriaceae	0.2%	-	-	-	-	-	-	4.9%	0.7%	-	-	-	0.6%	-
f__Enterobacteriaceae	3.7%	2.0%	-	-	0.3%	-	-	0.9%	2.5%	-	0.8%	5.6%	3.5%	-
f__Lactobacillaceae	3.6%	17.6%	-	-	-	-	-	0.1%	-	0.1%	0.1%	-	-	-
f__Leuconostocaceae	0.3%	-	-	-	-	-	-	-	-	-	-	-	-	-
f__Sphingomonadaceae	3.6%	4.4%	-	-	0.4%	0.1%	0.1%	5.4%	1.7%	-	0.2%	3.1%	3.5%	-
f__Acetobacteraceae	-	-	-	-	-	-	-	-	-	52.0%	0.2%	-	0.8%	2.5%
f__Geodermatophilaceae	-	-	-	-	-	-	-	-	30.2%	-	-	-	-	-
Others	27.7%	14.4%	4.6%	4.2%	3.2%	2.9%	3.0%	37.5%	20.7%	3.0%	7.3%	15.8%	27.6%	2.6%

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Table S3. OTUs shared among the three types of substrates (fresh fruits, fermented fruit and brines) considering sampling time and industry all together. Only OTUs assigned at genus level and *Enterobacteriaceae* and *Lactobacillaceae* families are shown.

p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae; g_Cyanothece
p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae; g_Sufflavibacter
p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae; g _Flavisolibacter
p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae; g__Bacillus
p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae; g_Faecalibacterium
p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__Rhodothermaceae;g__Salinibacter
p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g_Chryseobacterium
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter
p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus
p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__Owenweeksia
p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Balneimonas
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Rhodovibrio
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Herbaspirillum
p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus
p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Ectothiorhodospiraceae;g__Halorhodospira
p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium
p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia
p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter
p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter
p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium
p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Micrococcus
p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Phenylobacterium
p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Erwinia
p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Weissella
p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Celerinatantimonas
p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae

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Table S4. OTUs shared in fruit samples among all the different sampling time considering the two industries together. Only OTUs assigned at genus level and *Enterobacteriaceae* and *Lactobacillaceae* families are shown.

p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__Rhodothermaceae;g_Salinibacter
p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Rhodovibrio
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus
p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus
p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium
p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium
p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Celerinatantimonas
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae
p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae

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Table S5. OTUs shared in brine samples among all the different sampling time considering the two industries together. Only OTUs assigned at genus level are shown.

p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus
p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae;g__Cyanothece
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium
p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__Rhodothermaceae;g_Salinibacter
p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus
p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium
p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia
p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus
p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__Owenweeksia
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella
p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Rhodovibrio
p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium
p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Celerinatantimonas

3.1.4. CAPÍTULO 4

Enhancement of the knowledge on fungal communities in directly brined Aloreña de Málaga green olive fermentations by metabarcoding analysis

PLOS | ONE

RESEARCH ARTICLE

Enhancement of the Knowledge on Fungal Communities in Directly Brined *Aloreña de Málaga* Green Olive Fermentations by Metabarcoding Analysis

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Abstract

Nowadays, our knowledge of the fungal biodiversity in fermented vegetables is limited although these microorganisms could have a great influence on the quality and safety of this kind of food. This work uses a metagenetic approach to obtain basic knowledge of the fungal community ecology during the course of fermentation of natural *Aloreña de Málaga* table olives, from reception of raw material to edible fruits. For this purpose, samples of brines and fruits were collected from two industries in Guadalhorce Valley (Málaga, Spain) at different moments of fermentation (0, 7, 30 and 120 days). The physicochemical and microbial counts performed during fermentation showed the typical evolution of this type of processes, mainly dominated by yeasts in apparent absence of *Enterobacteriaceae* and *Lactobacillaceae*. High-throughput barcoded pyrosequencing analysis of ITS1-5.8S-ITS2 region showed a low biodiversity of the fungal community, with the presence at 97% identity of 29 different fungal genera included in 105 operational taxonomic units (OTUs). The most important genera in the raw material at the moment of reception in the industry were *Penicillium*, *Cladosporium*, *Malassezia*, and *Candida*, whilst after 4 months of fermentation in brines *Zygotorulaspora* and *Pichia* were predominant, whereas in fruits were *Candida*, *Penicillium*, *Debaryomyces* and *Saccharomyces*. The fungal genera *Penicillium*, *Pichia*, and *Zygotorulaspora* were shared among the three types of substrates during all the course of fermentation, representing the core fungal population for this table olive specialty. A phylogenetic analysis of the ITS sequences allowed a more accurate assignment of diverse OTUs to *Pichia manshurica*, *Candida parapsilosis/C. tropicalis*, *Candida diddensiae*, and *Citeromyces nyonensis* clades. This study highlights the existence of a complex fungal consortium in olive fermentations including phytopathogenic, saprofitic, spoilage and fermentative genera. Insights into the ecology, identification and quantification of fungi species in

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olive fermentation will facilitate the design of new strategies to improve the quality and safety of this fermented vegetable.

Introduction

The cultivation of *Olea europaea* tree has a dual purpose, the production of both edible table olives and olive oil, which depends of the olive variety used. When refer table olives, we are talking of a traditional fermented vegetable with many centuries of history in the Mediterranean basin, with a worldwide production which nowadays exceeds 2.5 million tons/year [1]. Green Spanish-style, Greek naturally black and ripe Californian styles are the most popular commercial table olive preparations [2]. However, in the last years, consumers are demanding more traditional and natural homemade seasoned olives. This is the case of *Aloreña de Málaga*, a traditional green olive preparation from Guadalhorce Valley (Málaga, Spain) with a Protected Designation of Origin (PDO) recognized by the European Union [3]. This olive cultivar has unique features which make them quite different from others: its fruits are characterized by an excellent flesh-to-stone ratio, a green–yellow color, a crispy firmness, and a peculiar mild bitter taste. Due to its low-to-moderate concentrations of bitter compounds, the processing does not include alkaline debittering. Thus, they are produced as natural olives and seasoned at the moment of packaging. The manufacturing process is carried out spontaneously by small and medium enterprises placed in, or very close to, the region of production [4].

Lactic acid bacteria (LAB) have an important role during fermentation of lye treated table olives [5]. These microorganisms produce lactic acid and bacteriocins by sugars consumption, contributing to the safe preservation of olives. However, in directly brined (natural) olives, yeasts are also relevant microorganisms coexisting with LAB during fermentation process, or even being the majority microorganisms if LAB are inhibited by the presence of phenolic compounds or the high salt and low pH levels obtained [2, 6]. Yeasts are unicellular eukaryotic microorganisms classified in Fungi kingdom, isolated from many foods and ubiquitous in nature. Their presence during table olive processing was reported in the earliest studies of this product [7–8]. In particular, they can play a double role acting as desirable (due to both technological and probiotic characteristics) or spoilage microorganisms (production of CO_2, unwanted odors/flavors, the consumption of lactic acid, the softening of fruits or clouding of olive brines) [9]. In the last years, diverse publications have emphasized the great importance that yeasts could have during olive fermentations [9–12].

Recently, diverse molecular methods have been used to identify the yeast species associated to Spanish style [13–14] and natural [11–12, 15–17] industrial olive fermentations. In the specific case of the *Aloreña de Málaga* olive cultivar, diverse authors have used a culture-dependent approach based in RFLP analysis of the 5.8- Internal Transcriber Spacer (ITS) region and sequencing of the D1/D2 domains of 26S rRNA gene to determine the yeast biota associated to this table olive specialty [13, 18–19]. However, the use of methods that rely on the cultivation of microorganism in selective media do not offer a complete profile of the microbial diversity that is present in olive ecosystem and only a small portion of the true microbial population is detected. For this reason, a culture-independent approach (PCR-DGGE) for the study of the yeast biodiversity in *Aloreña de Málaga* fermentations was also used [20]. All these studies were performed exclusively with brines and they did not take into consideration the study of the fungal population adhered to olive surface, which is finally the food intake by consumers.

High-throughput sequencing has emerged as a new culture-independent tool to quantitatively investigate the biodiversity of microbial communities in foods in order to look at

dominant as well as minor microbial populations, gaining at the same time information of the fermentative process and the microbiota of raw materials [21–22]. It also has revolutionized the field of food microbial ecology via more accurate identification of microbial taxa without the need for cultivation-dependent methods, showing a huge previously unknown microbial diversity no revealed by conventional methodologies. In the specific case of table olive fermentations, recently this powerful methodology has been used for the study of the bacterial biodiversity adhered to the surface of diverse Italian olive varieties using the 16S rRNA encoding gene as marker [23–24], but no attention was paid in those studies on fungal communities. Unfortunately, information based in high-throughput sequencing of ITS region to determine the fungal population dynamic in fermented vegetables is still scarce. The use of next generation sequencing to decipher a fungal ecosystem requires a different approach, targeting the ITS region, a non-coding DNA sequence situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome. The ITS database is somewhat less advanced than for the 16S rDNA gene, but it is gradually improving in the last years [25].

The aim of this study was to use a metagenetic approach to obtain basic knowledge of the changes in the fungal communities through raw material until end of fermentation of PDO *Aloreña de Málaga* table olives, to rationally assess the influence of industry of origin, ecological niche and fermentation time on the population dynamics of these eukaryotic microorganisms. Insight into the fungal life of table olive fermentation will allow us to obtain valuable information of the fermentation process and the structure of fungal community for the design of new strategies to improve the quality and safety of this fermented vegetable.

Materials and Methods

Type of samples

Samples were obtained from industrial fermentations of PDO *Aloreña de Málaga* table olives during October 2014 to January 2015. Fruits were harvested at green maturation stage, washed to remove impurities, cracked and directly brined in a 110 g/L NaCl solution in fermentations vessels with 220 L capacity (130 kg fruits). When necessary, fermentation vessels were supplemented with new brine of 120 g/L NaCl and 13 g/L citric acid. Two different industries labelled as COP (UTM ETRS89 coordinate 333969–4066126) and TOL (UTM ETRS89 coordinate 331261–4061750) located at Guadalhorce Valley (Malaga, Spain) were sampled. Both industries are separated by a distance of almost 5.3 km by air but they produce the same denomination of product (traditional PDO *Aloreña de Málaga* olives). Samples were obtained from two different fermentations vessels in each industry from fermentation brines (B) and fruits (F) at the time of reception in the factory (fresh fruit, FF) and after 7 (initial stage of fermentation), 30 (minimum time of brining contemplated by PDO *Aloreña de Málaga* normative) and 120 (moment of packaging established by demand) days of fermentation. Table 1 shows the references of the 28 samples analyzed in the present study and their origin.

Monitoring of the industrial fermentations

The analyses of brines for NaCl, pH, titratable and combined acidity were carried out using the routine methods described for table olives [2]. For the counts of microbial populations (*Enterobacteriaceae*, yeasts and LAB) in both brine and fruits, samples were spread in selective media according to methods previously described [26]. Counts were expressed as $\log_{10}$ cfu/mL for brines, or $\log_{10}$ cfu/g for olives.

Table 1. Number of sequences and OTUs analyzed, observed diversity and estimated sample coverage for ITS rRNA amplicons from olives fermentations at two industries.

Sample	Matrix	Industry	Time	Number of reads	Number of OTUs	Good's coverage	Chao1[a]	Richness[a]
FF-COP-0-A	Fresh Fruit	COP	0 months (0 days)	2275	23	99.78	20.03	18.8
FF-COP-0-B	Fresh Fruit	COP	0 months (0 days)	1377	23	99.71	23.33	20.3
F-COP-0-A	Fruit	COP	0 months (7 days)	1516	40	99.67	39.02	35.7
F-COP-0-B	Fruit	COP	0 months (7 days)	1353	32	99.78	32.82	29.5
F-COP-1-A	Fruit	COP	1 month (30 days)	2095	7	99.95	6.20	6.1
F-COP-1-B	Fruit	COP	1 month (30 days)	1933	15	99.64	16.55	10.8
F-COP-4-A	Fruit	COP	4 months (120 days)	2153	30	99.86	29.58	26.0
F-COP-4-B	Fruit	COP	4 months (120 days)	2126	30	99.62	27.00	21.0
B-COP-0-A	Brine	COP	0 months (7 days)	736	50	98.64	57.99	50.0
B-COP-0-B	Brine	COP	0 months (7 days)	853	46	99.06	51.32	44.6
B-COP-1-A	Brine	COP	1 month (30 days)	1603	6	99.94	5.70	5.7
B-COP-1-B	Brine	COP	1 month (30 days)	1584	9	99.81	8.40	7.0
B-COP-4-A	Brine	COP	4 months (120 days)	3303	32	99.82	30.63	25.4
B-COP-4-B	Brine	COP	4 months (120 days)	1790	25	99.83	23.42	22.2
FF-TOL-0-A	Fresh Fruit	TOL	0 months (0 days)	1152	31	98.96	38.87	26.8
FF-TOL-0-B	Fresh Fruit	TOL	0 months (0 days)	1370	26	99.56	27.06	22.7
F-TOL-0-A	Fruit	TOL	0 months (7 days)	1391	22	99.71	21.88	19.9
F-TOL-0-B	Fruit	TOL	0 months (7 days)	1861	28	99.62	23.20	19.9
F-TOL-1-A	Fruit	TOL	1 month (30 days)	923	15	99.57	19.85	14.5
F-TOL-1-B	Fruit	TOL	1 month (30 days)	1725	18	99.65	17.69	14.3
F-TOL-4-A	Fruit	TOL	4 months (120 days)	2511	31	99.76	27.13	21.4
F-TOL-4-B	Fruit	TOL	4 months (120 days)	1822	27	99.73	27.46	22.6
B-TOL-0-A	Brine	TOL	0 months (7 days)	2072	13	99.86	10.39	9.3
B-TOL-0-B	Brine	TOL	0 months (7 days)	1755	48	99.32	48.37	37.7
B-TOL-1-A	Brine	TOL	1 month (30 days)	903	15	99.34	25.30	14.0
B-TOL-1-B	Brine	TOL	1 month (30 days)	1696	14	99.82	15.04	11.1
B-TOL-4-A	Brine	TOL	4 months (120 days)	4186	24	99.83	19.48	15.3
B-TOL-4-B	Brine	TOL	4 months (120 days)	4389	31	99.82	33.80	18.9

[a] Values were estimated after rarefaction to 730 sequences. A and B stands for the two different fermentation vessels sampled in each industry.

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Extraction of DNA from olive samples and pyrosequencing

All samples were treated in the same day for DNA extraction from solid or liquid olive matrix. In the case of brine samples, a volume of 50 mL was taken from fermentation vessels and spun at 9,000 x *g* for 20 min at 5°C. Then, the pellet was washed twice in saline solution (9 g/L NaCl). In the case of fruit samples, 20 g of pulp was homogenized with 50 mL of saline solution in a stomacher and the aqueous phase was spun to get a pellet with same conditions describe above. DNA isolation was done using the PowerFood® Microbial DNA Isolation Kit (MoBio, Carlsbad, Calif.) according to the manufacturer instructions. Purified DNA samples (~10 ng/μL) were stored at –20°C until use.

DNA extracts obtained from the 28 collected samples (4 from FF, 12 from F and 12 from B; see Table 1) were used for the fungal community analysis. This way, the 28 DNA samples were submitted to PCR-amplification of the ITS1-5.8S-ITS2 of rRNA gene. Three independent 20-μL PCRs were performed for each sample using a tailed PCR approach. For the first PCR round the ITS1F (5'-*CTTGGTCATTTAGAGGAAGTA A*-3') primer that specifically amplify fungal sequences [27] linked to universal M13/pUC forward (5'-*GTTGTAAAACGACGGC*

CAGT-3') sequence and the ITS4 (5'-*TCCTCCGCTTATTGATA TGC*-3') primer linked to universal M13/pUC reverse (5'-*CACAGGAAACAGCTATGAC C*-3') sequence (M13F-*ITS4* and M13R-*ITS1F*) were used [28]. The cycling program for the first round of PCR was an initial denaturation step of 10 min at 95°C, followed by 35 cycles of 1 min denaturation at 95°C, 45 s annealing at 55°C and 1 min extension at 72°C, and a final 10 min extension step at 72°C followed by a 4°C soak. Then, second PCR reactions were performed using a 10x dilution of the first PCR product with the fusion forward primer of the Lib-L consisting of the A-adaptor sequence 5'-*CCATCTCATCCCTGCGTGTCTCCGAC*-3' followed by the 4-base calibration sequence 5'-*TCAG*-3', a 10-base MID oligonucleotide to differentiate each of the 28 samples and the 20-base M13F/pUC forward oligonucleotide. The reverse fusion primer consisted of the Lib-L B-adaptor sequence 5'-*CCTATCCCCTGTGTGCC TTGGCAGTC*-3' followed by the 4-base calibration sequence, and the 20-base M13/pUC reverse oligonucleotide. The cycling program for this second round of PCR was an initial denaturation step of 5 min at 95°C, followed by 15 cycles of 20 s denaturation at 95°C, 20 s annealing at 60°C and 30 s extension at 72°C, and a final 5 min extension step at 72°C followed by a 4°C soak. HPLC-purified oligonucleotides were synthesized by TIB MOLBIOL (Berlin, Germany). All PCR reactions were run in a T100TM Thermal Cycler (Bio-rad, Madrid Spain) using the FastStart High Fidelity Polymerase (Roche Diagnostics GmBH, Mannheim, Germany) and conditions recommended by the manufacturer for pyrosequencing analysis for long amplicons. The PCR products were purified twice with AgencourtH AMPureH XP PCR purification system (Agencourt Bioscience Co., Beverly, MA, USA) and quantified using the Quant-iT dsDNA Assay kit High sensitivity (Invitrogen, Carlsbad, CA, USA) and a fluorometer (BioTek Instruments, Winooski, VT, USA). Subsequently, all samples from each run were pooled in equimolar concentrations and purified again twice with AgencourtH AMPureH XP PCR. Pools of the 28 samples were diluted to obtain a total of $1x10^5$ copies/μL and emulsion PCR was performed with the Lib-L kit (454 Life Sciences) according to manufacturer's instructions for long reads. DNA positive beads were enriched, counted on the GS Junior Bead Counter, and loaded onto a picotiter plate and run in a 454 Life Sciences (Roche) Junior platform according to the standard platform protocols for long sequencing runs.

Bioinformatic analysis of pyrosequencing reads

Sequences were processed and analyzed according to procedures previously described [29] using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (version v1.9.1. http://qiime.sourceforge.net/) with default parameters unless otherwise noted. Sequences were first screened for quality using the following parameters: minimum quality score of 25, minimum sequence length of 200 bp, maximum length 1,000 bp, and no ambiguous bases in the entire sequence or mismatches in the primer sequence. Any sequences not meeting these parameters were excluded from downstream analyses. Sequences were then sorted by barcode into their respective samples and the barcode and primer sequences were removed. Chimeras were removed and operational taxonomic units (OTUs) were clustered *de novo* (pick_de_novo_otus.py script) using USEARCH at 97% identity. Taxonomy was assigned to the OTUs against the UNITE version 7 database for ITS sequences [30] available at http://qiime.org/home_static/dataFiles.html and then compared manually to that obtained against NCBI database (last access 25/02/2016). GI identifiers of found best-match sequences were used to extract taxonomy from NCBI taxonomy database. Singleton OTUs were filtered out of the entire dataset to reduce the noise caused by PCR or sequencing error, and we also discarded those OTUs that were present in less than 10% of samples. Sequences are available at the Sequence Read Archive of Genbank under BioProject ID PRJNA317749.

Differences between fungal communities were calculated in QIIME using rarefaction curves of alpha-diversity indexes including estimates of community richness (such as the Chao1 estimator, Richness or the observed number of OTUs present in each sample, and Good's coverage). Rarefaction analysis was performed using rarefied OTU tables (rarefied to 730 sequences; the lowest number of reads obtained for any of the 28 DNA samples analyzed to control for differing depths of sequencing across the samples), 100 replications, and cut-offs of 97% sequence similarity. Beta-diversity Bray-curtis distance matrices were built after subsampling all samples to an even depth of 730 sequences per sample. Taxonomic abundances within each identified Phylum to genus level were visualized using Krona hierarchical data browser [31]. Principal coordinates analysis (PCoA) was also performed on the Bray-curtis dissimilarity matrices to visualize the differences between the sample types, and visualized using the KiNG graphics program (http://kinemage.biochem.duke.edu/software/king.php). Statistical significance of differences in alpha-diversity were performed with QIIME using a nonparametric two sample t-test with 999 Monte Carlo permutations on number of observations and Chao1 and in beta-diversity a nonparametric ANOSIM tests on Bray-Curtis distance matrices (ITS).

Phylogenetic analysis

For more accurate assignation of OTUs at species levels, ITS sequences of all OTUs assigned to *Pichia*, *Candida*, *Debaryomyces*, and *Lodderomyces* were aligned using the MEGA software version 5.05 [32] with those acquired from GenBank database for diverse reference type strains of *Candida*, *Pichia*, *Debaryomyces*, *Lodderomyces*, *Citeromyces*, *Wickerhamomyces*, *Yamadazyma*, and *Meyrozyma* species, previously curated in diverse published works of phylogeny and related with table olive processing [9]. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA software version 5.05 only with ITS sequences >300 bp. The evolutionary distance data were calculated from Kimura's two-parameter model with the maximum-likelihood method [33]. Gaps and missing data were treated as complete deletions. Confidence limits were estimated from bootstrap analysis (1000 replicates).

Results

Verification of the fermentation process

The fermentation process of traditional *Aloreña de Málaga* table olives was followed during four months in two different factories by physicochemical and microbiological analyses. The evolution of the main physicochemical characteristics showed a similar behavior in both industries, except salt concentration which was slightly lower in TOL industry at the onset of fermentation (67 g/L) than in COP (80 g/L). The profile of pH and combined acidity in brines was kept practically constant during all fermentation process, with mean values of 4.4 and 0.10 Eq/L, respectively. On the contrary, the salt concentration and titratable acidity increased through fermentation process, reaching similar final values in both industries with 95 g/L and 0.60%, respectively. These data show the acidified and salted environment that olive fermentations represent for microorganisms.

Regarding microbial counts, yeasts were the predominant microorganism detected during the study. Thereby, they increased their population levels during fermentation process, with counts higher in TOL than in COP industry for much time of fermentation (Fig 1). However, after four months of study, this fungal group reached practically the same population level in both industries, with 5.0 $\log_{10}$ cfu/mL in brines, and 4.5 $\log_{10}$ cfu/g in fruits. *Enterobacteriaceae* and LAB were below limit of detection (<1.2 $\log_{10}$) during all fermentation process, in both fruits and cover brines.

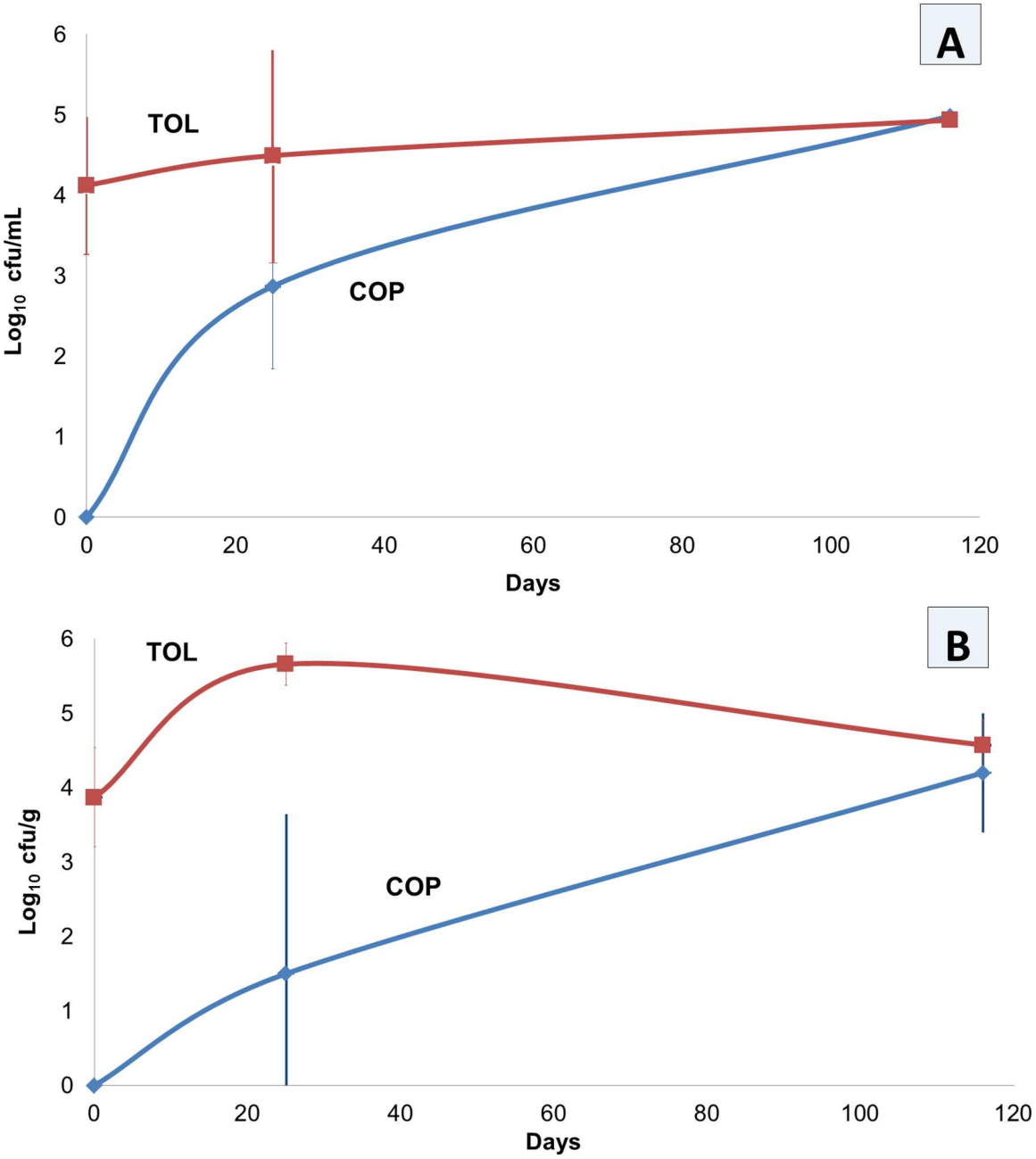

Fig 1. Yeast counts in brines (A) and fruits (B) during industrial fermentation process of *Aloreña de Málaga* table olives. TOL and COP stands for the two different industries analyzed in this work.

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Table olive fungal community structure

The complete panel of amplicons of the ITS-PCR products obtained from the 28 samples analyzed yielded a total of 70,983 raw sequences, with a mean of 1,928 reads per sample and average length of 554 bp. After denoising of data for poor quality sequences, we recovered 54,005 high-quality ITS rRNA gene sequences with an average of 1,823 sequences per sample. From those, it was obtained a total of 52,453 sequences that could be appropriately classified into OTUs with a mean of 1,873 classifiable sequences per sample. Table 1 shows the total number of reads obtained in the different samples, as well as the number of OTUs assigned.

According to the analysis of the complete ITS data set, the structure of the global fungi community composition showed big differences between the three types of substrates analyzed (fresh fruit, fermented fruit and brine samples) (S1–S3 Figs). The analysis showed that the fungal phyla *Ascomycota* was the most represented in the three substrates, with 99% of sequences in fermented fruit and brine samples, while the phylum *Basidiomycota* was also represented with 4% of sequences in fresh fruits (with the family *Malasseziaceae*). Within *Ascomycota* phylum, in fresh fruits the classes *Saccharomycetes*, *Dothideomycetes*, and *Eurodomycetes* were practically represented in the same proportions (S1 Fig), whilst in brines and fermented fruit samples the *Saccharomycetes* was clearly the predominant class (S2 and S3 Figs). At family taxa, *Saccharomycetaceae* and *Pichiaceae* were the most important families in both brine and fermented fruit samples. On the contrary, the families *Mycosphaerellaceae* and *Trichocomaceae*, together with *Candida* (included in *Incertae sedis*), were found in higher proportions in fresh fruits (S1–S3 Figs).

ITS sequences were associated with a total of 105 OTUs belonging to 29 different fungal genera, with an average of 25 observed OTUs (6 to 50) per sample (see Table 1). Only 1.58% of total sequences could not be assigned at genus level. Despite the high number of taxa identified, few genera accounted for most reads. The frequency of fungi genera changed with the type of substrate, during the fermentation process and between factories (Fig 2). This way, the genera *Candida*, *Cladosporium*, *Penicillium*, and *Malassezia* accounted for 95% of sequences in fresh fruits. On the contrary, in the fermented fruits, the majority of genera at the onset of fermentation (7 days) were *Zygotorulaspora* (>75% sequences), while at 30th day were *Pichia* and *Zygotorulaspora*, and at the end of fermentation process (120 days) dominated *Penicillium*, *Candida*, *Saccharomyces*, and *Debaryomyces* were prevalent, in this order. In the fermentation brine samples, at the beginning of fermentation, *Candida*, *Cladosporium*, and *Saccharomyces* were the genera found in higher proportions (>90% sequences), at 30th day were *Zygotorulaspora* and *Pichia*, whilst at the end of fermentation process the dominant genera were *Zygotorulaspora*, *Pichia*, and *Penicillium* (Fig 2). Although there were some differences in the fungal community composition between both industries, those were sown mainly at the end of the fermentation process and in brine samples Fig 2).

Biodiversity of the fungal community

The Venn diagrams show that the number of unique and shared fungal OTUs changed with the type of substrate and during the course of fermentation (Fig 3). Taking into consideration only the type of substrate, the highest number of fungal OTUs was observed for brines samples (102), followed by fermented olives (92) and fresh fruits (59). A total of 47 OTUs (44.8%) represented the core fungal population for the three types of substrates, whilst fermented fruit and brine samples sharing a higher number of OTUs (89, 85.0%). Only 4 OTUs were unique for brine samples (Fig 3A), belonging to genera *Pyrenochaeta*, *Alternaria*, *Bionectria*, and *Candida* (*C. tartivorans*), where there were not specific OTUs for fruit samples. S1 Table shows the OTUs assigned by metabarcoding analysis at genera and species levels shared among the three types of substrates analyzed. Among the fungi species present in all substrates (raw material, fermented fruit and brine samples) we can foreground *Penicillium paneum*, *Aspergillus niger*, *Candida diddensiae*, *Saccharomyces cerevisiae*, *Zygotorulaspora mrakii*, *Debaryomyces hansenii*, and *Lodderomyces elongisporus*, together with genus *Pichia*. Looking exclusively at fruit samples, 12 fungal OTUs (11.9%) were shared by all sampling times including fresh fruits. The number of OTUs increased with fermentation time (i.e., 45 OTUs for F-0, 47 OTUs for F-1, and 59 OTUs for F-4) (Fig 3B). Fermented fruits after 30 days of fermentation (F-1) showed the highest number of unique OTUs (Fig 3B). S2 Table shows the OTUs assigned at genera and

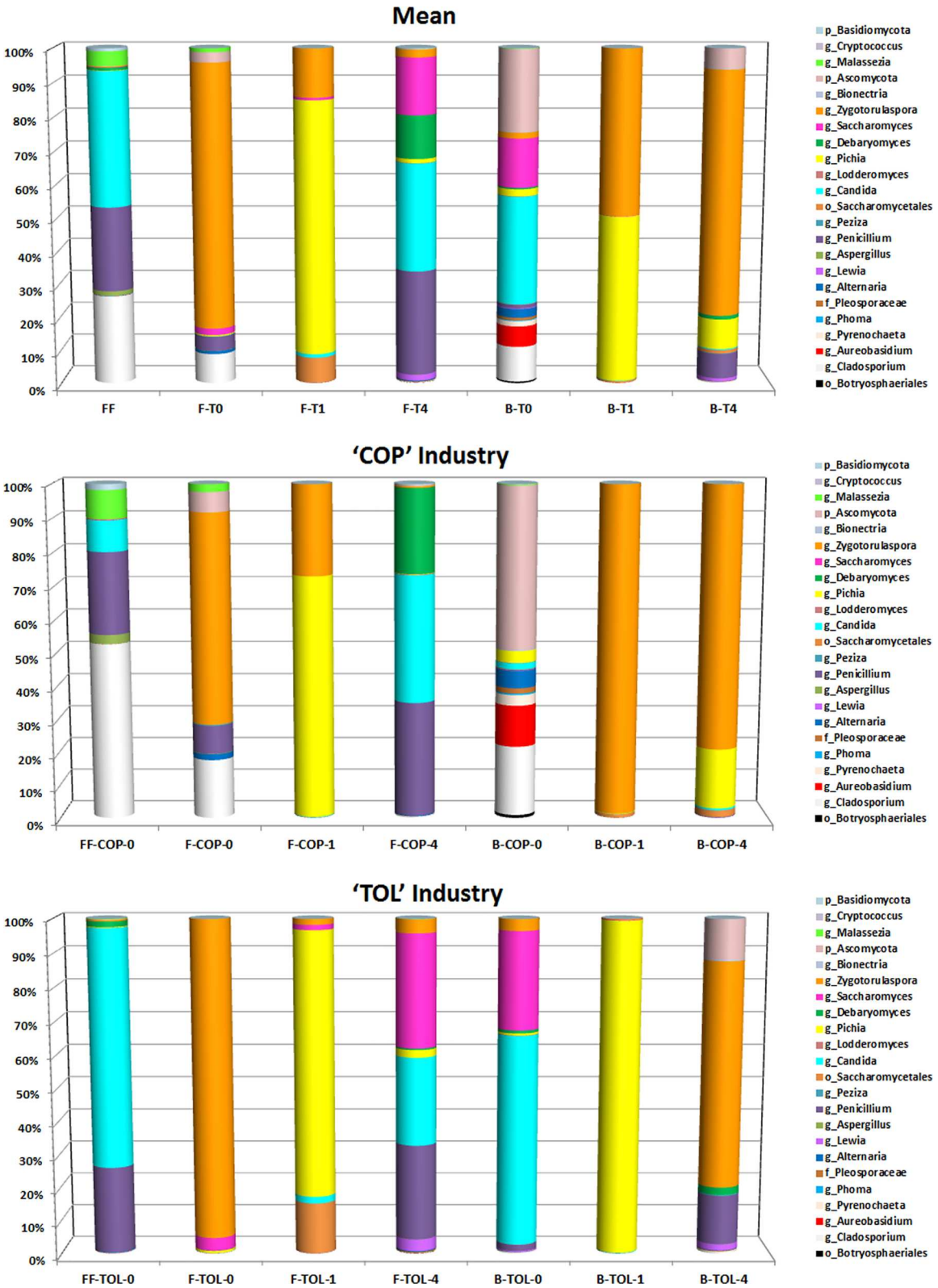

Fig 2. Relative abundance (%) of fungi at genera or family level obtained by pyrosequencing analysis throughout the fermentation process. The different industries (COP and TOL) are shown together (upper graph) and independently (middle and bottom graphs). FF, F, and B stands for fresh fruits, fermented fruits and fermentation brines, respectively, while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation, respectively).

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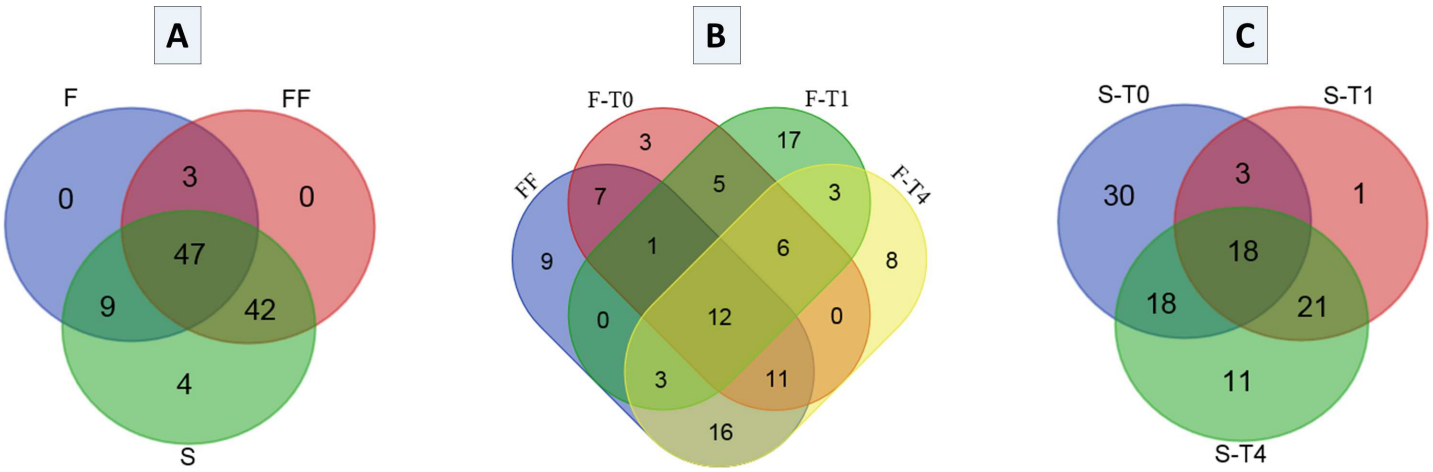

Fig 3. Venn diagrams showing the number of unique and shared OTUs among substrates (A), sampling times in fruits (B) and sampling times in cover brines (C). FF, F, and B stands for fresh fruits, fermented fruits and cover brines, respectively, while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation, respectively).

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species level shared among the fruits in all sampling times. This way, the species *P. paneum*, *S. cerevisiae*, and *Z. mrakii* were present in the fruits during all the course of fermentation, together with genera *Pichia* and *Cladosporium*. In brine samples, a total of 18 fungal OTUs (17.6%) were shared among all times with the brines samples at 7 days of fermentation showing the highest number of total and unique OTUs (Fig 3C). S3 Table shows the OTUs assigned at genera and species levels shared among the brine samples in the different sampling time. *Z. mrakii* and *D. hansenii* were the only species present in the brine during all the course of fermentation, accompanied by the genera *Pichia* and *Penicillium*. In summary, the fungi genera *Penicillium*, *Pichia*, and *Zygotorulaspora* were shared among the three types of substrates assayed during all the course of fermentation (see S1–S3 Tables), representing the core fungal population for *Aloreña de Málaga* table olive fermentations.

The fungal community was also analyzed using rarefaction curves and richness estimator (Chao1 index). The Chao1 index varied from 5.70 (one of the brine samples obtained from COP industry after 30 days of fermentation) to 57.99 (one of the brine samples obtained from the same factory at the onset of fermentation) (Table 1). The rarefaction analysis assigned to 97% of OTUs similarity showed the achievement of the saturation zone for all samples, suggesting that a number of fungal reads of 730 per sample was satisfactory to obtain a good coverage despite the diversity of sequencing depth between samples (Table 1; Fig 4). Thus, there was a satisfactory coverage of the fungal diversity for all the samples analyzed with Good's coverage values above 98.6% for all samples (Table 1). Alpha-diversity rarefaction curves indicated that globally there were no significant differences ($P>0.05$) between industries with most differences occurring between fruit and brines samples and during the fermentation process ($P<0.05$), with similar pattern for both alpha-diversity indexes (Chao1 and Richness) (in Fig 4 only data for Richness are shown). For fruit samples, there were no significant ($P>0.05$) differences in alpha-diversity, with a slight trend to decrease alpha-diversity after 30 days of fermentation. For brine samples, there were significant differences ($P<0.05$) during the fermentation process, with the lowest alpha-diversity values occurring 30 days after the fermentation started in both industries and the highest alpha-diversity values at the beginning of fermentation (Fig 4).

Finally, beta-diversity analysis based in PCoA of Bray-Curtis distance matrices of ITS sequences segregated olive fruits samples unprocessed (FF) and at the beginning of the

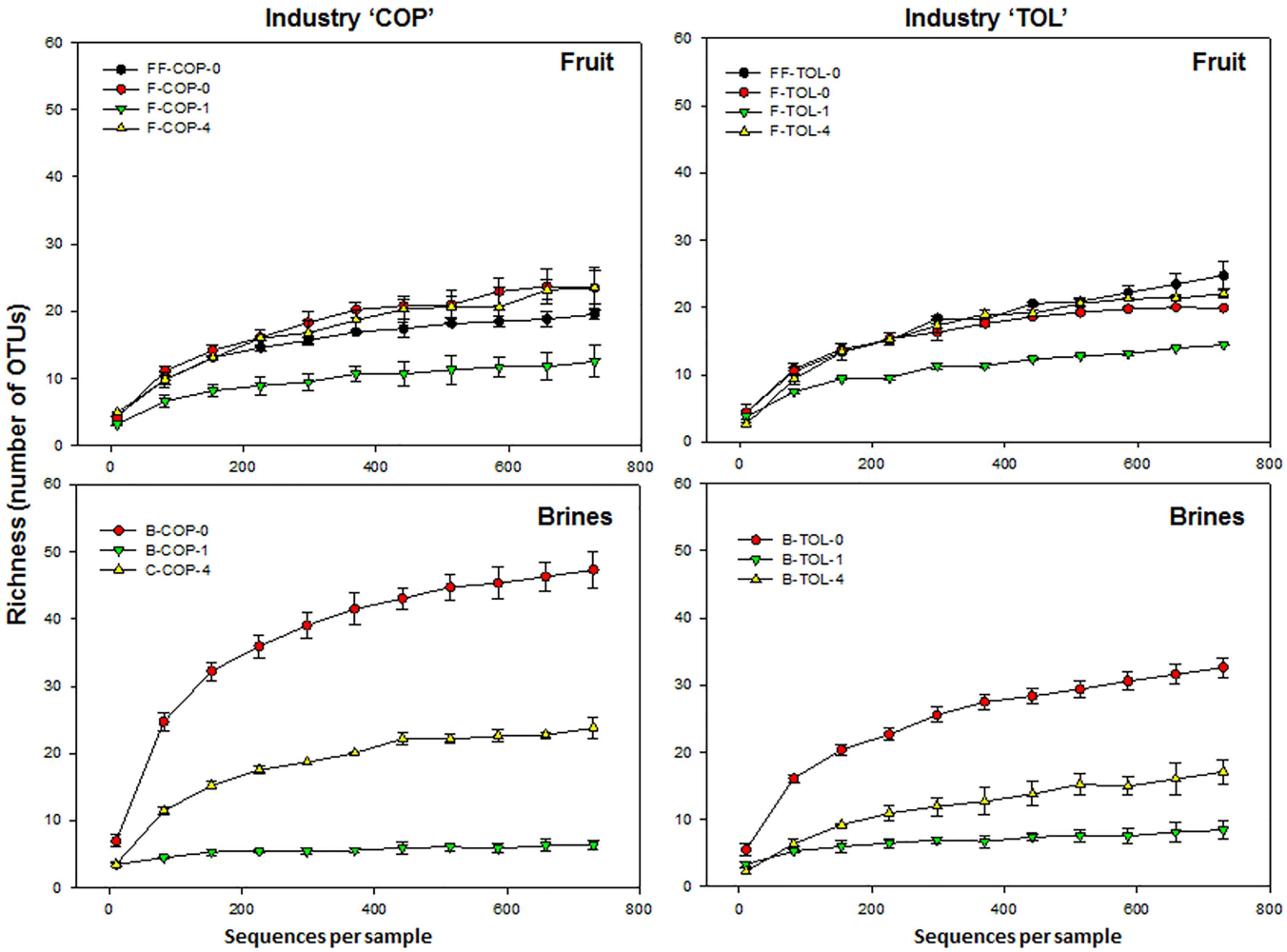

Fig 4. Rarefaction curves of fungal community for the different industries and substrates. FF, F, and B stands for fresh fruits, fermented fruits and cover brines, respectively, while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation, respectively). Data shown are the mean of two fermentation vessels sampled at each industry.

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fermentation process (F-0) from the rest of samples along PC1 (30% of total variance) irrespectively of the industry, while samples at 30th and after 120th days of fermentation were mainly separated along PC2 axis (22% of variance). On the contrary, all fermented fruit and brines samples for both industries tended to group together at 30th (with one exception; sample from fermentation vessel A in TOL industry) while after 120th days of fermentation samples from both industries were clearly differentiated (with one exception; sample B-COP-4-A) pointing out that the changes occurring during the fermentation process (time) were the main drivers of fungal community composition (Fig 5). Thus, ANOSIM test indicated that there were not statistical significant differences ($P<0.05$) only among the Unweighted UniFrac distances when comparing samples among the different sampling times.

Phylogenetic assignment of relevant genera

A total of 35 OTUs, assigned initially by the metagenetic analysis as *Candida* spp. (16 OTUs), *Pichia* (15 OTUs), *Debaryomyces* (2 OTUs) and *Lodderomyces* (2 OTUs), were subjected to phylogenetic assignment with the ITS sequences obtained from GenBank for diverse reference

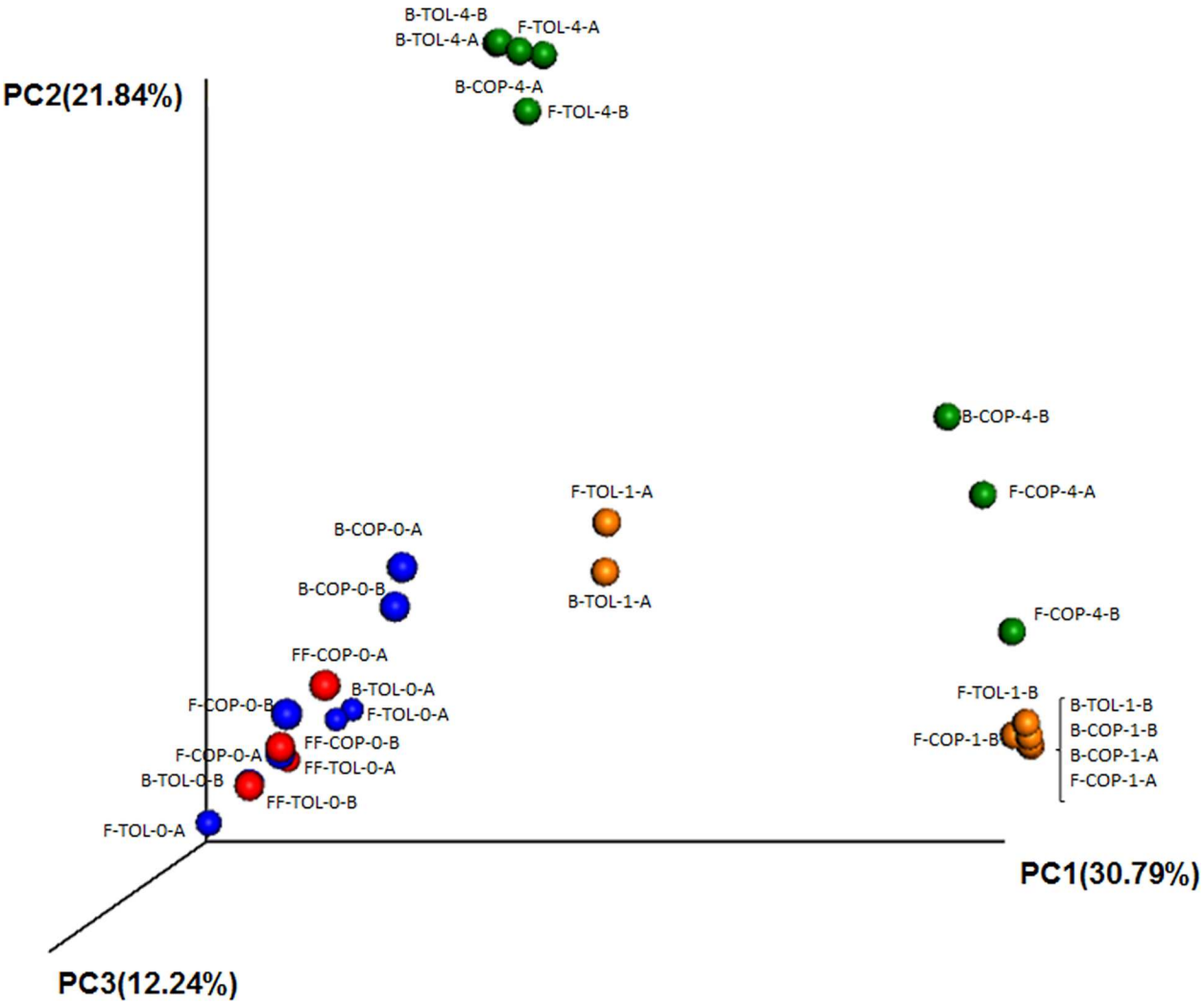

Fig 5. Unweighted UniFrac analysis based in principal coordinates analysis of ITS sequences obtained from different samples. FF, F, and B stands for fresh fruits, fermented fruits and brines, respectively, TOL and COP stands for different industries, 0, 1 and 4 stands for the different sampling times (0, 1, and 4 months of fermentation, in blue, orange and green colors, respectively), while and A and B stands for different fermentation vessels sampled in each industry.

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type strains of related species. Fig 6 shows the phylogenetic tree obtained after application of maximum-likelihood method with Kimura 2-parameters. Two major clades can be distinguished; one of them included most of the *Pichia* OTUs together with the reference type strains of *Pichia membranifaciens*, *P. manshurica*, *P. fermentans*, *P. kluyveri*, and *P. kudriavzevii*. In the case of OTUs 0, 21, 28, 32, 61, 74, 82, 94, 108, and 121, the metagenetic approach was only able to assign them at genus level, but the phylogenetic study showed a close relation of those OTUs with the reference strain of *P. manshurica*. On the contrary, the OTUs 33, 53 and 90, albeit were included in the *Pichia* clade confirming the assignation carried out initially against the UNITE database, but could not be closely clustered with any of the type strains of *Pichia* included in the phylogenetic analysis which might indicate they are new taxa (or sequences are not available for comparison in the ITS database). The other large clade was mainly formed by OTUs initially assigned to *Candida*, *Debaryomyces*, and *Lodderomyces*. Thus, OTUs 6, 47, and 147 initially assigned as *Candida nyonensis* were phylogenetically related with the type strains

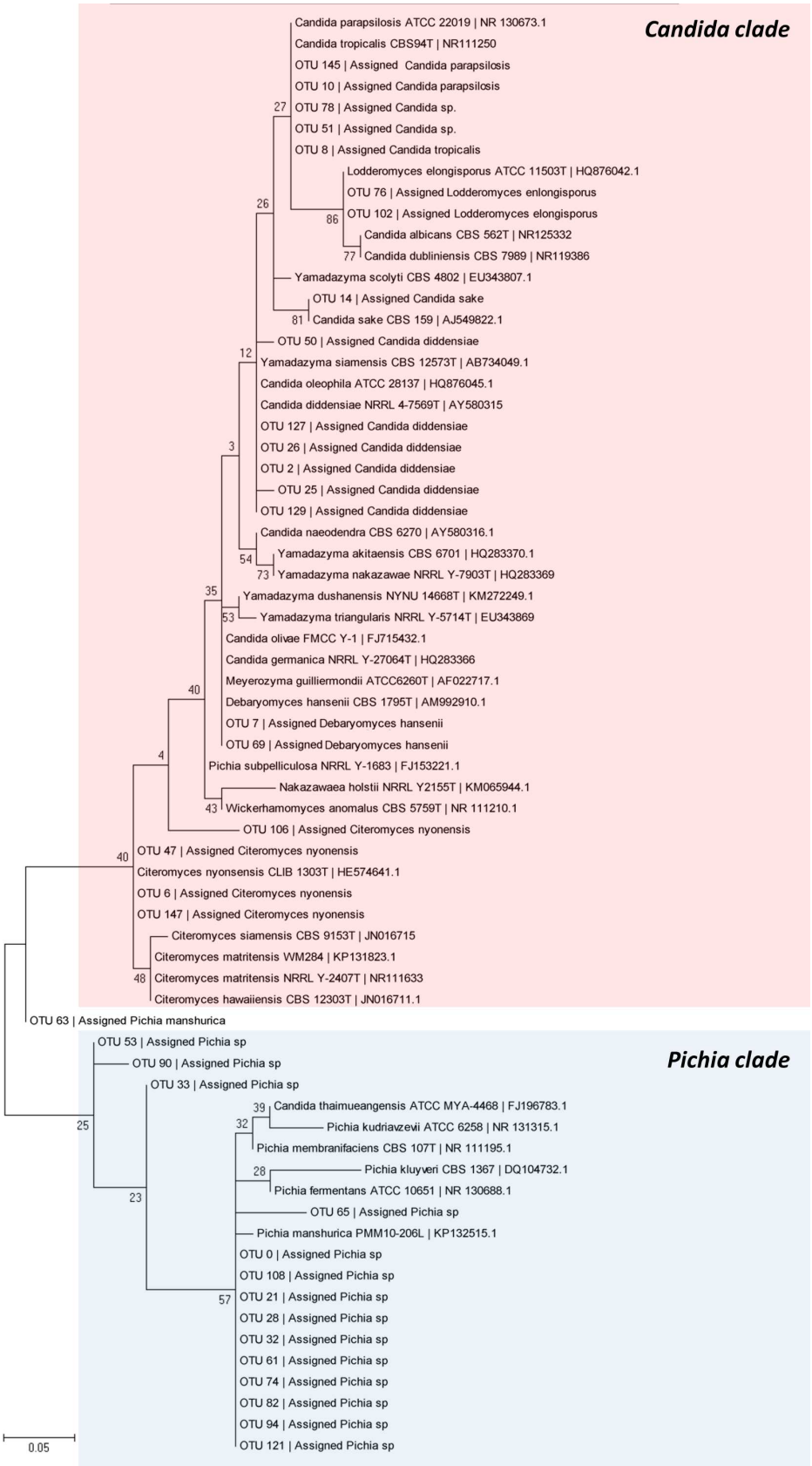
Candida clade
Candida parapsilosis ATCC 22019 | NR 130673.1
Candida tropicalis CBS94T | NR111250
OTU 145 | Assigned Candida parapsilosis
OTU 10 | Assigned Candida parapsilosis
27
OTU 78 | Assigned Candida sp.
OTU 51 | Assigned Candida sp.
OTU 8 | Assigned Candida tropicalis
Lodderomyces elongisporus ATCC 11503T | HQ876042.1
OTU 76 | Assigned Lodderomyces enlongisporus
26
OTU 102 | Assigned Lodderomyces elongisporus
86
Candida albicans CBS 562T | NR125332
77
Candida dubliniensis CBS 7989 | NR119386
Yamadazyma scolyti CBS 4802 | EU343807.1
OTU 14 | Assigned Candida sake
81
Candida sake CBS 159 | AJ549822.1
OTU 50 | Assigned Candida diddensiae
12
Yamadazyma siamensis CBS 12573T | AB734049.1
Candida oleophila ATCC 28137 | HQ876045.1
Candida diddensiae NRRL 4-7569T | AY580315
OTU 127 | Assigned Candida diddensiae
3
OTU 26 | Assigned Candida diddensiae
OTU 2 | Assigned Candida diddensiae
OTU 25 | Assigned Candida diddensiae
OTU 129 | Assigned Candida diddensiae
Candida naeodendra CBS 6270 | AY580316.1
54
Yamadazyma akitaensis CBS 6701 | HQ283370.1
73
Yamadazyma nakazawae NRRL Y-7903T | HQ283369
35
Yamadazyma dushanensis NYNU 14668T | KM272249.1
53
Yamadazyma triangularis NRRL Y-5714T | EU343869
Candida olivae FMCC Y-1 | FJ715432.1
Candida germanica NRRL Y-27064T | HQ283366
Meyerozyma guilliermondii ATCC6260T | AF022717.1
40
Debaryomyces hansenii CBS 1795T | AM992910.1
OTU 7 | Assigned Debaryomyces hansenii
OTU 69 | Assigned Debaryomyces hansenii
4
Pichia subpelliculosa NRRL Y-1683 | FJ153221.1
Nakazawaea holstii NRRL Y2155T | KM065944.1
43
Wickerhamomyces anomalus CBS 5759T | NR 111210.1
OTU 106 | Assigned Citeromyces nyonensis
40
OTU 47 | Assigned Citeromyces nyonensis
Citeromyces nyonsensis CLIB 1303T | HE574641.1
OTU 6 | Assigned Citeromyces nyonensis
OTU 147 | Assigned Citeromyces nyonensis
Citeromyces siamensis CBS 9153T | JN016715
Citeromyces matritensis WM284 | KP131823.1
48
Citeromyces matritensis NRRL Y-2407T | NR111633
Citeromyces hawaiiensis CBS 12303T | JN016711.1
OTU 63 | Assigned Pichia manshurica
Pichia clade
OTU 53 | Assigned Pichia sp
OTU 90 | Assigned Pichia sp
OTU 33 | Assigned Pichia sp
39
Candida thaimueangensis ATCC MYA-4468 | FJ196783.1
25
32
Pichia kudriavzevii ATCC 6258 | NR 131315.1
Pichia membranifaciens CBS 107T | NR 111195.1
28
Pichia kluyveri CBS 1367 | DQ104732.1
23
Pichia fermentans ATCC 10651 | NR 130688.1
OTU 65 | Assigned Pichia sp
Pichia manshurica PMM10-206L | KP132515.1
OTU 0 | Assigned Pichia sp
OTU 108 | Assigned Pichia sp
57
OTU 21 | Assigned Pichia sp
OTU 28 | Assigned Pichia sp
OTU 32 | Assigned Pichia sp
OTU 61 | Assigned Pichia sp
OTU 74 | Assigned Pichia sp
OTU 82 | Assigned Pichia sp
OTU 94 | Assigned Pichia sp
OTU 121 | Assigned Pichia sp
0.05

Fig 6. Phylogenetic placement of the OTUs assigned initially by the metabarcoding analysis as *Pichia*, *Candida*, *Debaryomyces*, and *Lodderomyces* genera, respect to diverse type strains of the genera *Candida*, *Pichia*, *Lodderomyces*, *Meyerozyma*, *Wickerhamomyces*, *Debaryomyces*, *Yamadazyma*, and *Citeromyces* related to table olive processing [9]. Their respective GenBank accession numbers are indicated in the phylogenetic tree. The analysis was performed with the ITS sequences and the maximum-likelihood method. Bar, 5 nucleotide changes per 100 nucleotides.

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of *Citeromyces nyonensis* (synonymous of *C. nyonensis*) and the *Citeromyces* clade. OTUs 8, 10, 51, 78, and 145 were related with the type strains of *C. parapsilosis* and *C. tropicalis* (OTUs 51 and 78 were only assigned initially as *Candida* spp.), whilst OTUs 2, 25, 26, 127, and 129 were related with the type strain of *C. diddensiae*. The closest species to the *Lodderomyces* OTUs, apart from the type strain ATTCC 11503T, were *C. albicans* and *C. dubliniensis*, whilst the *D. hansenii* sequences (anamorph state *C. famata*) were related with *Meyerozyma guilliermondii* (anamorph state *C. guilliermondi*), *Candida olivae*, and *Candida germanica* type strains. Only OTU 63, initially assigned as *Pichia manshurica*, show a dubious position in the phylogenetic tree. Thus, the phylogenetic analysis confirms many of the initial assignment made against the UNITE database, and also related diverse OTUs that initially were assigned only at genus level with the type strains of certain *Pichia* and *Candida* species.

Discussion

The main physicochemical and microbiological changes which occurred during fermentation process of *Aloreña de Málaga* olives were related with a slight salt and titratable acidity increase. Both parameters were very similar in both industries and can be considered as the usual ones at the end of the fermentation process of this specialty of natural, cracked, green olives. The flavor and aroma of fermented olives were also tested by a training panel, not detecting the presence of abnormal taste or smells and resulting in the typical product (data not shown). Hence, the samples obtained for metabarcoding analysis can been considered as representative of this type of process, dominated by yeasts because of the high salt and low pH levels obtained [2].

Apart from table olives [23–24], metagenetic analysis has been also used to investigate the changes in bacterial communities in diverse vegetables in brines such as asparagus [34], cucumbers [35], and kimchi [36]. However, not special attention has been paid to the study of fungal communities in vegetables. These microorganisms are especially relevant in directly brined olives due to the inhibition of LAB by the presence of phenolic compounds [5–6, 9]. The only study on this matter was recently carried out by HiSeq Illumina sequencing to determine the fungal communities in serofluid dish, a traditional food in the Chinese culture made from vegetables by fermentation [37]. *Candida* and *Sporpachydermia* were the dominant genera found in that product. Thus, according to our knowledge, there is no available information regarding metagenetic yeast data in the specific case of table olives and vegetables in general. The metagenetic studies of fungal communities in food and beverages are scarce compared to bacteria, with the exception of some products such as fermented yak milk [38], kefir grains and milks [39], kombucha [40], sake [41], cocoa bean [42] and cheese [43] fermentations which all use the ITS as target region.

A great disadvantage of the ITS regions for metabarcoding analysis is related to taxonomical differentiation of phylogenetically related species for some genera that may have similar sequences. Hence, the databases and bioinformatics analysis give reliable microbial identification up to the level of the genus, as occurs in this paper, and they are less confident when used for assignment of fungi to the species level. In addition, a significant part of deposited ITS sequences are not updated or curated, following the latest studies in fungal taxonomy. For this

reason, in certain occasions a phylogenetic assignment with reference sequences is performed as a second step for accurate identification [44]. The problematic of differentiating closely related species using short DNA barcodes and pyrosequencing analysis with genus-specific primers was also recently discussed for the oomycete *Phytophthora* [45]. Nevertheless, in our study, this methodology allowed identification of initially assigned OTUs at genus level to *P. manshurica* and *C. parapsilosis/C. tropicalis*, and also the confirmation of the species *C. diddensiae*, *D. hansenii*, *L. elongisporus*, and *C. nyonensis*. Our data shows the need and usefulness of this dual approach for accurate and correct identification at species level of relevant fungal genera. However, despite the above limits and biases, the ITS region is widely accepted as the official fungal DNA barcode marker because it can be easily amplified and sequenced by different molecular approaches and provides enough resolution for most fugal species.

Amplicons were analysed with QIIME using a high quality filtering set up in order to minimize the impact of sequencing errors and achieve a reliable identification of fungi population. Despite the high number of taxa identified, few genera accounted for most reads. This way, a conspicuous part of sequences detected were associated with well-known fermentative yeasts. In particular, the genera *Zygotorulaspora* and *Pichia* were found in the raw material, fresh fruits and brines during all the course of fermentation (at least in one of the industries), representing 55.43% of the total of sequences obtained. Thus, they can be considered as the most representative fungi genera of this table olive specialty. The species *Z. mrakii* and *P. manshurica* were the most important species included in these genera. *Candida* (with 12.3% of total sequences) and *Saccharomyces* (9.13%) were also some genera detected with certain frequency during the fermentation process. The genera *Candida*, *Pichia*, *Zygotorulaspora*, and *Saccharomyces* have been previously described by molecular methods as usual components of the fungal population present during elaboration of *Aloreña de Málaga* [13, 18–20] and other natural table olive elaborations [11–12, 15–17]. Apart from sugar consumption, diverse species of these genera have relevant technological and probiotic characteristics with application in table olive processing, such as production of killer toxins, aromatic compounds, degradation of bitter glucosides, lipase and esterase activities, production of vitamins, biodegradation and bioapsortion of mycotoxins, etc. [9]. The presence of these fermentative yeasts was most habitual during the course of fermentation, except *Candida* spp. which was also detected at high frequencies in the fruits at the moment of reception in the industry.

In our study, the methodology used has also allowed the identification of diverse non-fermentative fungi genera which could play other roles during table olive processing. Some of these genera have been previously described as phytopathogenic microorganisms in olive and other plants, such as *Alternaria*, *Phoma*, *Pyrenochaeta*, and *Bionectria* [44]. However, all them together only represented 0.31% of total sequences, mainly detected at the early stages of fermentation. Thus, their influence on the fermentative process must be scarce. *Cladosporium* and *Aeurobasidium* spp. were also detected in the *Aloreña* samples, mainly in fresh fruits or at the beginning of fermentation, with 5.34% of the total sequences. Both genera were also previously detected by pyrosequencing analysis in leaves, flowers and fruits of olives, suggesting a possible competitive action against the fungal plant pathogens described above [44]. Finally, the study shows also the presence of *Penicillium* (practically in all samples) and *Aspergillus*, both of them considered undesirable microorganisms because of their ability to produce mycotoxins and cellulose and xylanase activities which can produce softening of fruits. Both spoilage genera have been previously described in different table olive processing in presence of oxygen [46–48] and represented the 8.09% of total sequences obtained. *Penicillium* spp. seems to be specially adapted to the fermentative process, because of their presence practically in all samples analyzed.

In summary, results obtained of the present work reveal the complex structure of the fungal community in natural table olive fermentations, from raw material to edible fruits. The fungal consortia showed to contain phytopathogenic, epiphytic, spoilage and fermentative microorganisms that can have a significant impact in the production of this table olive specialty, typically dominated by yeasts. Also, although some differences were found between both industries, the global diversity patters were maintained. We consider that this type of studies are needed to enhance our knowledge of the microbiology of table olive fermentations and fungi in foods. Further studies are also necessary to determine the specific role played by these genera on the quality and safety of table olives.

Supporting Information

S1 Fig. Global taxonomic abundances (%) of fungi community from Phylum to genus level in the fresh fruit samples at the moment of reception in the industry. The different industries and sampling times were considered together for elaboration of the graphs.
(HTML)

S2 Fig. Global taxonomic abundances (%) of fungi community from Phylum to genus level in the fermented fruit samples. The different industries and sampling times were considered together for elaboration of the graphs.
(HTML)

S3 Fig. Global taxonomic abundances (%) of fungi community from Phylum to genus level in the brine samples. The different industries and sampling times were considered together for elaboration of the graphs.
(HTML)

S1 Table. OTUs shared among the three types of substrates (fresh fruits, fermented fruit and brine samples) considering sampling time and industry factors all together. Only OTUs well assigned at genus and species levels by metabarcoding analysis are shown.
(DOC)

S2 Table. OTUs shared in fruit samples among all the different sampling time considering the two industries together. Only OTUs well assigned by metabarcoding analysis at genus and species levels are shown.
(DOC)

S3 Table. OTUs shared in brine samples among all the different sampling time considering the two industries together. Only OTUs well assigned at genus and species levels by metabarcoding analysis are shown.
(DOC)

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Supervision: FNAL BBL.

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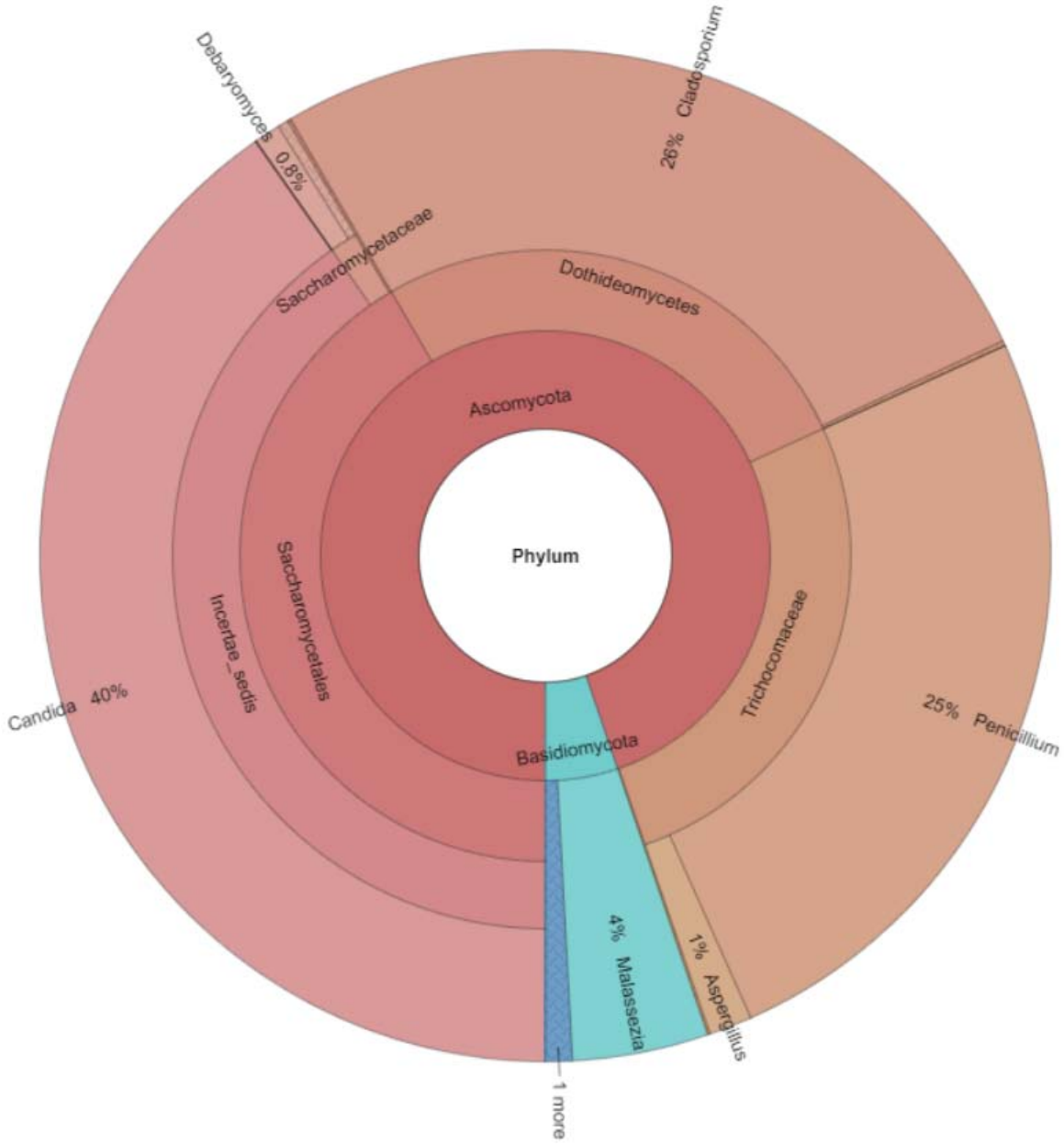
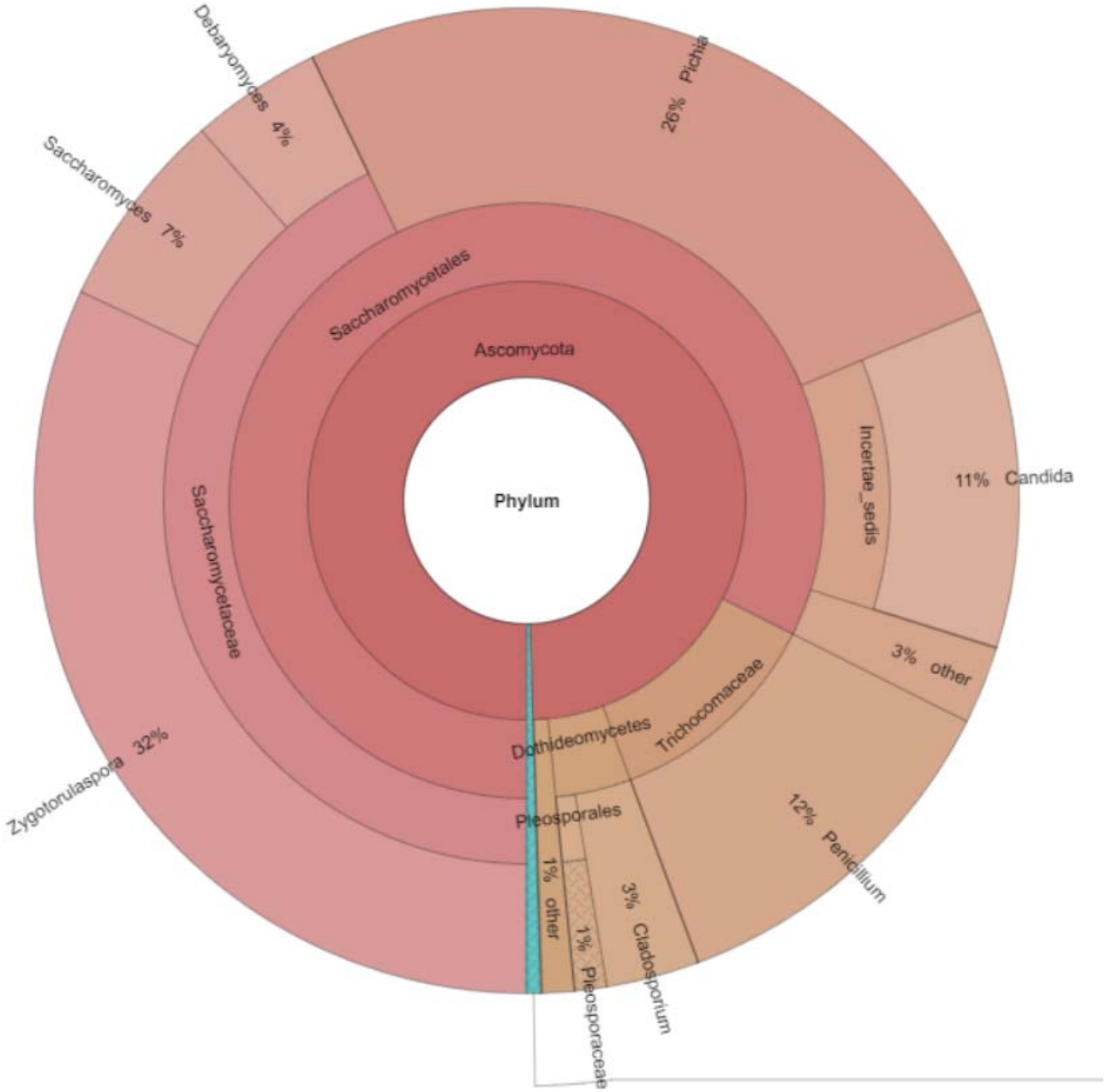
Supplementary material to: *Enhancement of the Knowledge on FungalCommunities in Directly Brined Aloreña de Málaga Green Olive fermentations by Metabarcoding Analysis*

S1 Fig. Global taxonomic abundances (%) of fungi community from Phylum to genus level in the fresh fruit samples at the moment of reception in the industry. The different industries and sampling times were considered together for elaboration of the graphs.



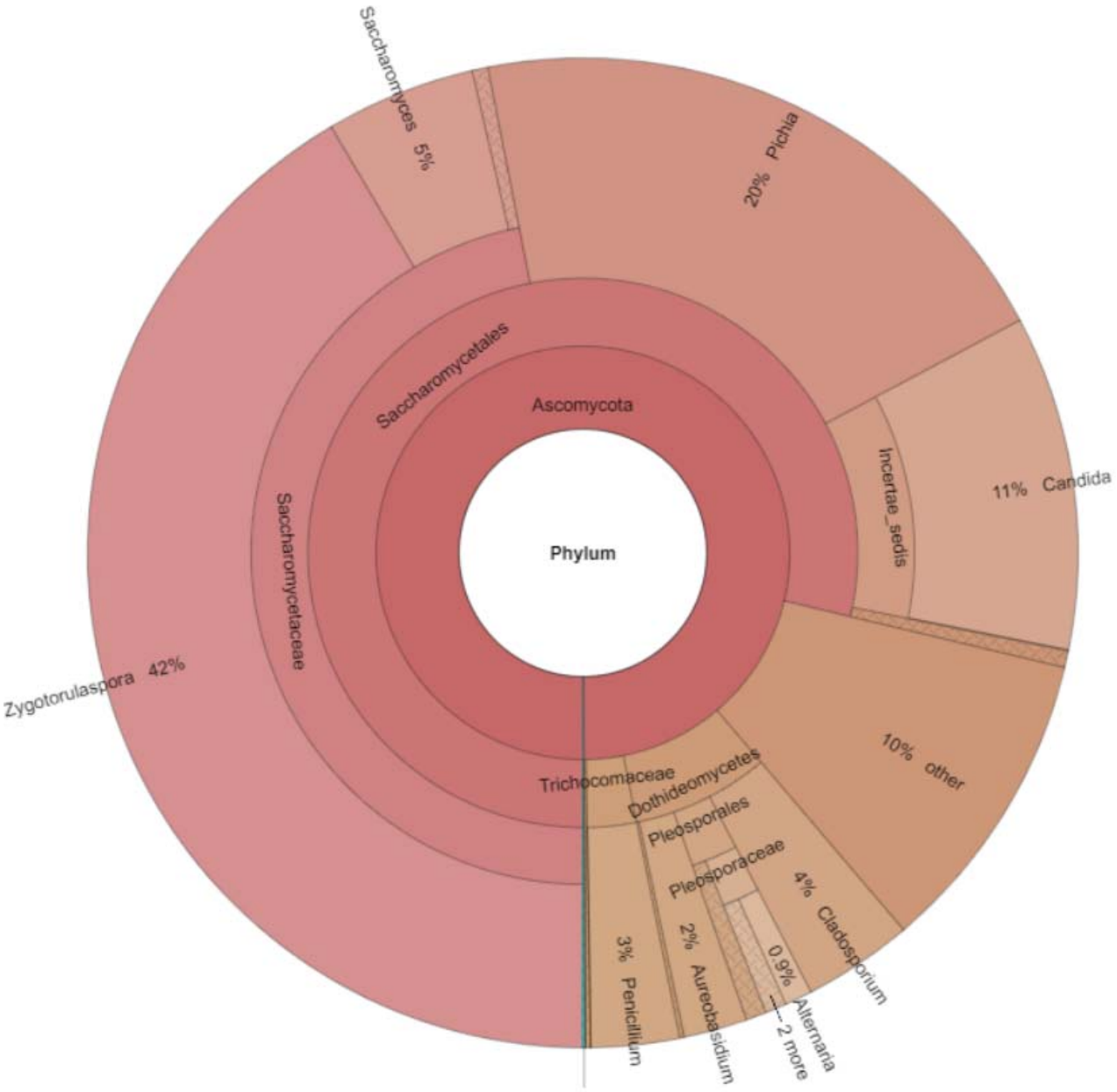
Supplementary material to: *Enhancement of the Knowledge on FungalCommunities in Directly Brined Aloreña de Málaga Green Olive fermentations by Metabarcoding Analysis*

S2 Fig. Global taxonomic abundances (%) of fungi community from Phylum to genus level in the fermented fruit samples. The different industries and sampling times were considered together for elaboration of the graphs.



Supplementary material to: *Enhancement of the Knowledge on FungalCommunities in Directly Brined Aloreña de Málaga Green Olive fermentations by Metabarcoding Analysis*

S3 Fig. Global taxonomic abundances (%) of fungi community from Phylum to genus level in the brine samples. The different industries and sampling times were considered together for elaboration of the graphs.



Supplementary material to: *Enhancement of the Knowledge on FungalCommunities in Directly Brined Aloreña de Málaga Green Olive fermentations by Metabarcoding Analysis*

S1 Table. OTUs shared among the three types of substrates (fresh fruits, fermented fruit and brine samples) considering sampling time and industry factors all together. Only OTUs well assigned at genus and species levels by metabarcoding analysis are shown.

p_Ascomycota;c_Eurotiomycetes;o_Eurotiales;f_Trichocomaceae; g_Penicillium; s_P. paneum
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Incertae_sedis;g_Candida; s_C. parapsilosis
p_Ascomycota;c_Dothideomycetes;o_Capnodiales;f_Mycosphaerellaceae;g_Cladosporium
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Incertae_sedis;g_Candida; s_C. diddensiae
p_Basidiomycota;c_Malasseziomycetes;o_Malasseziales;f_Malasseziaceae;g_Malassezia
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Saccharomyces; s_S. cerevisiae
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Zygotorulaspora; s_Z. _mrakii
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Pichiaceae;g_Pichia
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Debaryomyces; s_D. hansenii
p_Ascomycota;c_Dothideomycetes;o_Dothideales;f_Dothioraceae;g_Aureobasidium
p_Ascomycota;c_Dothideomycetes;o_Pleosporales;f_Pleosporaceae;g_Alternaria
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Incertae_sedis;g_Lodderomyces; s_L. elongisporus
p_Ascomycota;c_Eurotiomycetes;o_Eurotiales;f_Trichocomaceae;g_Aspergillus; s_A. niger

S2 Table. OTUs shared in fruit samples among all the different sampling time considering the two industries together. Only OTUs well assigned by metabarcoding analysis at genus and species levels are shown.

p_Ascomycota;c_Eurotiomycetes;o_Eurotiales;f_Trichocomaceae;g_Penicillium; s_P. paneum
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Saccharomyces;s_S. cerevisiae
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Zygotorulaspora; s_Z. mrakii
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Pichiaceae;g_Pichia
p_Ascomycota;c_Dothideomycetes;o_Capnodiales;f_Mycosphaerellaceae;g_Cladosporium
p_Ascomycota;c_Eurotiomycetes;o_Eurotiales;f_Trichocomaceae;g_Penicillium

S3 Table. OTUs shared in brine samples among all the different sampling time considering the two industries together. Only OTUs well assigned at genus and species levels by metabarcoding analysis are shown.

p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Pichiaceae;g_Pichia
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Zygotorulaspora; s_Z. mrakii
p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Debaryomyces; s_D. hansenii
p_Ascomycota;c_Eurotiomycetes;o_Eurotiales;f_Trichocomaceae;g_Penicillium

3.1.5. CAPÍTULO 5

Microbiological and physicochemical changes in natural green heat-shocked Aloreña de Málaga table olives

frontiers in Microbiology

ORIGINAL RESEARCH
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Microbiological and Physicochemical Changes in Natural Green Heat-Shocked *Aloreña de Málaga* Table Olives

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Preserving the highly appreciated natural freshness of *Aloreña de Málaga* table olives and preventing their progressive darkening during processing is a major challenge. In this work, heat-shocked (60°C, 5 min) fruits were processed according to the three denominations referred to in the Protected Designation of Origen (cured, fresh green, and traditional) and their characteristics compared with those that followed the habitual industrial process (controls). The results revealed that the effects of the heat treatment on the evolution of pH, titratable acidity, salt, sugar, organic acid, ethanol content, texture, and color of fruits as well as on microbial populations (yeasts and lactic acid bacteria) were slight in the case of the fresh green and cured presentations. However, the differences between heat-shocked and its control were remarkable in the traditional process. Notably, the heat treatment favored lactic acid fermentation, retention of the green appearance of the fruits, stability during packaging, and led to the highest sensory evaluation. The metagenomic analysis carried out at the end of the fermentation revealed the presence in all samples of three genera (*Lactobacillus*, *Pediococcus,* and *Celerinatantimonas*) which encompassed most of the sequences. The number of *Lactobacillus* sequences was statistically higher ($p \geq 0.05$) in the case of traditional heat-shocked fruits than in its control.

Keywords: heat treatment, olive packaging, sensory evaluation, table olives, metagenomic analysis

INTRODUCTION

Table olives are a major component of the Mediterranean diet and culture. Nowadays, they constitute one of the most important fermented vegetables in the world, with a production which exceeds 2.4 million tons/year (International Olive Council [IOC], 2016). Green Spanish-style, Greek naturally black, and ripe Californian styles are among the most popular and well-known table olive commercial presentations in the world (Garrido-Fernández et al., 1997).

However, in the last years, consumers have demanded more traditional and natural homemade-style elaborations. This is the case of *Aloreña de Málaga*, a table olive speciality processed as natural green olives under a Spanish Protected Designation of Origin (PDO) recognized by the European Union (DOUE, 2012). Their peculiar characteristics are related

to the production area (climate, edaphology, and geographical location in the Guadalhorce Valley, Málaga, Spain). Therefore, their products are quite different from other green natural table olives. *Aloreña de Málaga* usually contains low-to-moderate concentrations of oleuropein (the main bitter compound of olives) and, for this reason, is not subjected to lye treatment for debittering. The speciality is seasoned with fennel, thyme, garlic, and pepper, which are frequently added during packaging, making the product rich in aroma. To preserve their typical organoleptic characteristics and highly valued freshness (green aspect), packages are not usually stabilized by pasteurization.

The PDO regulation includes three different denominations (López López and Garrido Fernández, 2006):

(i) Cured *Aloreña de Málaga* olives (CA). The harvested fruits are placed directly in brine (5–6% NaCl, 10,000 L fermentation vessels) where they undergo a full fermentation for a minimum of 90 days. Then, the olives are progressively cracked, seasoned and packaged according to demand.

(ii) Fresh Green (FG) *Aloreña de Málaga* olives. The product is characterized by the immediate cracking after harvesting. Then, the fruits are brined in a 10–11% NaCl solution in plastic drums (220 L volume), where they should remain for at least 3 days. After this period, the partially debittered olives are seasoned and packaged or, otherwise, stored in the same containers in chilled chambers (8°C). Under these conditions, the fruits retain their green appearance for several months.

(iii) Traditional *Aloreña de Málaga* olives (TA). In this case, just after harvesting, the fruits are cracked and brined in plastic drums (200 L volume) in a 10–11% NaCl solution. Then, the olives are stored for at least 20 days before commercialisation. During this period, the fruits undergo a partial fermentation, where progression and partial green color degradation depend on the storage time. Finally, the olives are seasoned and packaged according to demand using similar conditions to the previous process (FG).

In general, the freshness appearance is an attribute highly appreciated in this table olive speciality. However, greenness progressively decreases as the fermentation, storage or packaging time is prolonged. At the same time, brine and surface color gradually brown. Several factors may contribute to these changes. The loss in green color could be due to the degradation of chlorophyll in the acidic medium of the brines (Gallardo-Guerrero et al., 2013). The browning could also be caused by the oxidation and polymerisation of polyphenols by the polyphenol oxidase (PPO) activity (Segovia-Bravo et al., 2009). As demonstrated by Arroyo-López et al. (2007), most of these changes are produced during storage. Consequently, several strategies for mitigating these adverse effects have been tested, such as the application of washings and protective carbon dioxide atmosphere (Arroyo-López et al., 2007). Other alternatives recently studied are the use of antioxidant compounds (ascorbic acid and sodium metabisulfite) or various mineral salts ($MgCl_2$ and $ZnCl_2$) (Arroyo-López et al., 2008; Gallardo-Guerrero et al., 2013). However, an entirely satisfactory solution is not yet available.

Heat-shocked olives was a convenient procedure for ridding the fruits of naturally occurring interfering and competitive microbial groups, but also made the olives highly fermentable (Etchells et al., 1966). Balatsouras et al. (1983) also reported a slight improvement in fermentability by means of a heat-shock treatment applied to *Conservolea* green olives. Recently, the European project Probiolives (FP7-SME, ID-243471) also included heat-shock as a method for enhancing green olive fermentability and contribute to the predominance of the potential probiotic starter culture. Results showed that heat-shocked (80°C for 10 min) olives led to final products with high acceptability, although the inoculum predominance depended on the strain assayed.

The present work investigates the effects of a previous mild heat-shock treatment of the fruits on the fermentation and packaging processes of *Aloreña de Málaga* table olives. The objective is the production of a better product than the commercial commodity with improved fresh appearance and stability while maintaining similar sensory attributes.

MATERIALS AND METHODS

Raw Material and Experimental Design

Aloreña de Málaga fruits at the green ripening stage were provided by a local farmer (Manzanilla Aloreña S.C.A., Alora, Málaga, Spain) during the 2015/2016 season (140–260 fruits/kg

TABLE 1 | Summary of the experimental design applied in the study.

Acronym	PDO denomination	Heat-shock application	Storage temperature (°C)	Brining conditions*
CA-C	Cured *Aloreña* (whole fruits)	No (control)	25	6.7 Na, 0.54 AA
CA-H	Cured *Aloreña* (whole fruits)	Yes	25	6.7 Na, 0.54 AA
FG-C	Fresh Green *Aloreña* (cracked fruits)	No (control)	8	15.8 Na
FG-H	Fresh Green *Aloreña* (cracked fruits)	Yes	8	15.8 Na
TA-C	Traditional *Aloreña* (cracked fruits)	No (control)	25	15.8 Na
TA-H	Traditional *Aloreña* (cracked fruits)	Yes	25	15.8 Na

**Na, NaCl concentration (%, w/v); AA, acetic acid (%, v/v). The heat-shock treatment consisted of dipping the fruits into a water bath at 60°C for 5 min just before brining. All treatments were run in duplicate.*

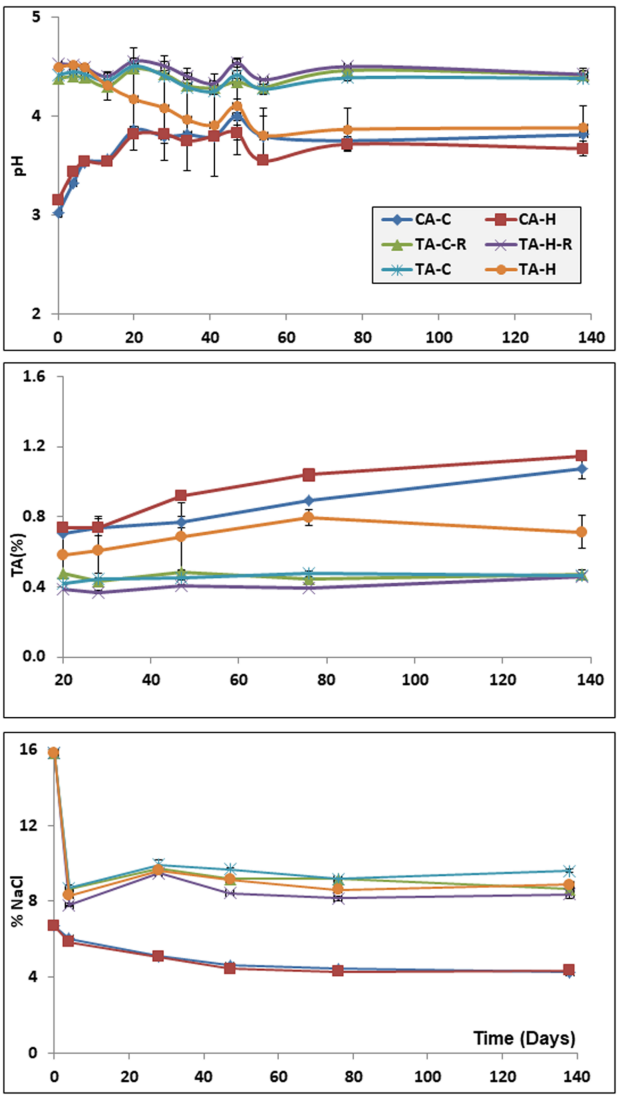

FIGURE 1 | Evolution of the pH **(upper)**, titratable acidity **(middle)** and salt content **(lower)** during fermentation in the diverse treatments. Error bars denote standard deviation calculated from duplicate fermentation vessels. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

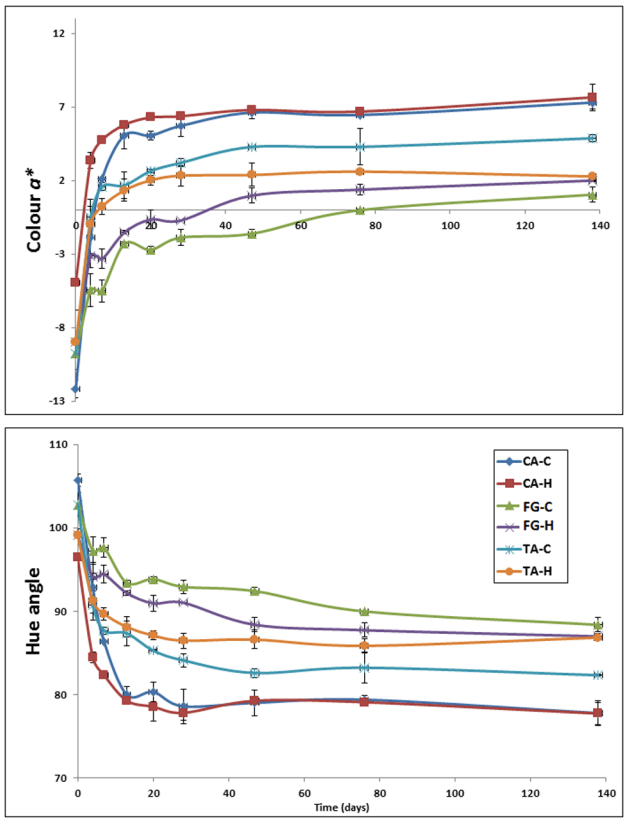

FIGURE 2 | Evolution of color parameters a^* **(upper)** and h_{ab} **(lower)** during fermentation in the diverse treatments. Error bars denote standard deviation calculated from duplicate fermentation vessels. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

size). The olives were processed at the pilot plant of the Instituto de la Grasa (CSIC, Seville) according to the three commercial denominations included in the PDO regulation. One part of them was prepared following the conditions applied by the industry (control treatments) while the rest were subjected to a mild-heat-shock treatment. **Table 1** summarizes the different treatments that constituted the experimental design. The heat-shock treatment was applied by dipping the fruits into a water bath at 60°C for 5 min just before brining. Then, the fruits were rapidly transferred into cool water and, after temperature equilibrium, placed in the fermentation vessels (5.3 kg of fruit and 3.8 L of brine). All treatments were run in duplicate, making a total of 12 containers. The fermentation process was monitored during 138 days.

Monitoring of the Fermentation Process

The analyses of the olive brine for pH, NaCl, titratable and combined acidity during the fermentation process were carried out by applying the usual methods described by Garrido-Fernández et al. (1997). The instrumental firmness and surface color of fruits analyses followed the methods described elsewhere (Chen et al., 2010; Bautista-Gallego et al., 2011). Color was measured using a BYKGardner Model 9000 Color-view spectrophotometer (MD, United States). Interference by stray light was minimized by covering the samples with a box with a matt black interior. Color was expressed as the CIE L^* (lightness), a^* (freshness, negative values indicate green while positive values are related to red tones), and h_{ab} (hue angle) parameters. The firmness of the olives was measured using a Kramer shear compression cell coupled to an Instron Universal Machine (Canton, MA, United States). The crosshead speed

TABLE 2 | Physicochemical characteristics of the diverse treatments at the end of the fermentation process (138 days).

Treatment	Texture (kN/100 g)	Glucose (g/l)	Sucrose (g/l)	Fructose (g/l)	Mannitol (g/l)	Total sugars (g/l)	Acetic acid (g/l)	Lactic acid (g/l)	Citric acid (g/l)	Ethanol (g/l)
CA-C	9.80 (0.24)^{d}	0.11 (0.01)^{a}	0.00 (0.00)^{a}	0.31 (0.00)^{a,b}	0.02 (0.01)^{a}	0.44 (0.03)^{a}	4.23 (0.01)^{b}	7.84 (0.82)^{b}	0.16 (0.00)^{b}	1.91 (0.09)^{b,c}
CA-H	8.31 (0.02)^{c}	0.16 (0.02)^{a}	0.00 (0.00)^{a}	0.26 (0.02)^{a}	0.02 (0.00)^{a}	0.43 (0.02)^{a}	3.99 (0.16)^{b}	10.08 (0.62)^{b}	0.00 (0.00)^{a}	1.27 (0.20)^{b}
FG-C	7.73 (0.17)^{b,c}	13.26 (0.16)^{e}	1.16 (0.13)^{c}	1.81 (0.14)^{d}	1.95 (0.36)^{b}	18.17 (0.20)^{d}	0.07 (0.05)^{a}	0.11 (0.05)^{a}	0.14 (0.01)^{b}	12.06 (0.36)^{a}
FG-H	6.26 (1.17)^{a}	5.54 (0.29)^{d}	0.14 (0.02)^{b}	0.43 (0.03)^{b}	2.45 (0.12)^{c}	8.57 (0.43)^{c}	0.08 (0.02)^{a}	0.18 (0.04)^{a}	0.15 (0.05)^{b}	11.62 (0.31)^{a}
TA-C	6.77 (0.60)^{a,b}	2.53 (0.10)^{b}	0.12 (0.02)^{a,b}	0.37 (0.00)^{a,b}	1.68 (0.01)^{b}	4.71 (0.09)^{b}	0.00 (0.00)^{a}	0.27 (0.05)^{a}	0.19 (0.01)^{b}	11.49 (0.33)^{a}
TA-H	6.54 (0.49)^{a,b}	3.93 (0.09)^{c}	0.01 (0.01)^{a,b}	0.07 (0.03)^{c}	0.29 (0.02)^{a}	4.31 (0.08)^{b}	1.07 (0.38)^{c}	10.19 (2.55)^{b}	0.15 (0.02)^{b}	2.33 (0.67)^{c}

Standard deviation from duplicate measurements in parentheses. Values followed by different superscript letters, within the same column, are statistically different ($p \leq 0.05$) according to LSD post hoc comparison test. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the Aloreña de Malaga cultivar.

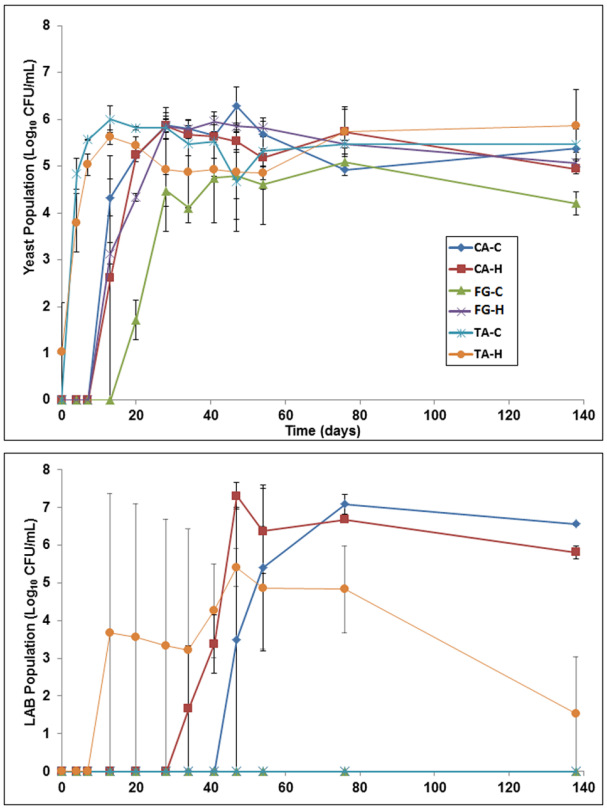

FIGURE 3 | Evolution of the yeast **(upper)** and lactic acid bacteria **(lower)** populations during fermentation in the diverse treatments. Error bars denote standard deviation calculated from duplicate fermentation vessels. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

was 200 mm/min. The firmness, expressed as kN/100 g flesh, was the mean of 10 replicate measurements, each of which was performed on three pitted olives. Individual reducing sugars (glucose, fructose, sucrose and mannitol), organic acids (acetic, lactic, and citric) and ethanol content were determined by HPLC according to the methods developed by Sánchez et al. (2000).

For the counts of the *Enterobacteriaceae,* yeasts and *Lactobacillaceae* populations in brine, samples drawn from the different treatments were spread onto selective media according to the methods described by Rodríguez-Gómez et al. (2015). Counts were expressed as $\log_{10}$ CFU/mL.

Metagenomic Analysis of Bacterial Populations

Microbial genomic DNA from olive and brine samples at the end of the fermentation process (138 days) was extracted as described by Medina et al. (2016) and sent to the Sequencing and Bioinformatic Service of FISABIO (Valencia, Spain) for bacterial metagenomic analysis. 16S rDNA gene amplicons were amplified following the 16S rDNA gene Metagenomic Sequencing

Library Preparation Illumina protocol. The gene-specific sequences used in this protocol target the V3 and V4 region of 16S rDNA gene (Klindworth et al., 2013). Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. The primer pair were: forward primer (5′-TCGT CGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNG GCWGCAG-3′) and reverse primer (5′-GTCTCGTGGGCTC GGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAA TCC-3′). A multiplexing step was performed using Nextera XT Index Kit (FC-131-1096). 1 μl of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size, the expected size on a Bioanalyzer trace should be ∼550 bp. The libraries were sequenced using a 2 × 300 pb paired-end run on a MiSeq Sequencer according to manufacturer's instructions (Illumina). Quality assessment was performed through the use of the prinseq-lite program (Schmieder and Edwards, 2011*)* by applying the following parameters: minimum sequence length of 50 bp, trim_qual_right of 20, trim_qual_type of mean and trim_qual_window of 20 bp.

A metagenomic analysis was performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (version1.9.1)[1]. Sequences were sorted by barcode into their respective samples. To calculate alpha diversity indexes, 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥97% sequence homology. Chimeric sequences were removed using ChimeraSlayer. All reads were classified into the lowest possible taxonomic rank using QIIME and the SILVA108 database. OTUs were assigned by means of uclust (Edgar, 2010) using the script pick_de_novo_otus.py. Alpha diversity was calculated through the alpha_diversity.py by script using different metrics (Chao, Observed Species, Shannon, Simpson, Good's coverage, PD whole tree) after performing a rarefaction analysis. Rarefied OTU tables to 6,500 sequences (lowest number of reads obtained) were used to obtain these alpha diversity metrics. OTU tables to Genus taxonomic level were exported in tab-delimited text format and analyzed using STAMP v2.0.1 (Parks and Beiko, 2010). An ANOVA/Tukey-Kramer (*post hoc*) test was run to elucidate the taxa association in the different grouping variables. The false discovery rate correction (Benjamini and Hochberg, 1995) was finally applied in all cases, and significant differences in taxa were only considered for $p \leq 0.05$ and a q-value below 0.3.

[1]http://qiime.sourceforge.net/

Packaging of Fruits

After 138 days of fermentation, the fruits obtained from the different treatments were washed (12 h) in tap water and then packaged in polyethylene terephthalate (PET) vessels (1.6 L volume). The packages were filled with 0.9 kg of olives, 16 g of seasoning material (a mixture of diced garlic, pepper strips, small pieces of fennel, and thyme) and 0.7 L of cover brine (3.0% NaCl). For each treatment, a total of 6 packages were obtained. Samples for physicochemical, microbiological, and sensory analysis were withdrawn on the 4th and 41st day of packaging.

Sensory Evaluation

The evaluation sheet developed by International Olive Council [IOC] (2010) for the estimation of acidic, salty, bitterness, hardness, and crunchiness attribute scores was used in the present study. Because of the specific sensory characteristics of this table olive speciality, other attributes such as darkening, appreciation of defects, and overall acceptability were also introduced into the evaluation sheet. The panel was composed of 14 expert members. Six of them were from the Instituto de la Grasa (CSIC) staff while the other 6 were from the industry. All of them were chosen because of their usual involvement in previous sensory analyses. Despite this, they were specifically trained (2 h for 2 weeks) for the sensory evaluation of the diverse commercial denominations of *Aloreña de Málaga* table olives. The evaluation sheet consisted of two sections. The first one was devoted to the sample and panelist identification while the second included the attributes to be evaluated, including a final question

TABLE 3 | Number of sequences and OTUs assigned (after removing chloroplast), diversity indexes, and estimated sample coverage for 16S (bacteria) amplicons according to treatments.

Sample	Matrix	Number of reads	Number of OTUs	Coverage	PD whole tree[a]	Chao1[a]	Simpson[a]	Shannon[a]
CA-C-B	Brine	51,667	176	97.90	7.63	615.31	0.34	1.49
CA-H-B	Brine	13,667	197	97.93	2.51	498.86	0.56	2.13
CA-C-E	Fruit	23,335	163	97.86	4.93	748.79	0.21	0.98
CA-H-E	Fruit	13,956	158	98.08	6.29	545.80	0.19	0.92
FG-C-B	Brine	31,582	192	97.95	3.40	557.30	0.56	2.17
FG-H-B	Brine	39,323	302	96.78	1.70	762.35	0.80	3.53
FG-C-E	Fruit	6,583	201	97.83	11.93	589.33	0.72	2.83
FG-H-E	Fruit	27,979	249	97.36	12.11	659.54	0.77	3.29
TA-C-B	Brine	18,888	225	97.43	9.87	765.46	0.47	2.08
TA-H-B	Brine	25,951	180	97.82	7.59	701.43	0.14	0.86
TA-C-E	Fruit	31,416	213	97.63	9.41	686.71	0.52	2.22
TA-H-E	Fruit	23,425	189	97.69	7.67	679.00	0.19	1.07

[a]*Values were estimated after rarefaction to 6,583 sequences. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the Aloreña de Malaga cultivar. B and E stand for samples obtained from brine or epidermis of fruits, respectively.*

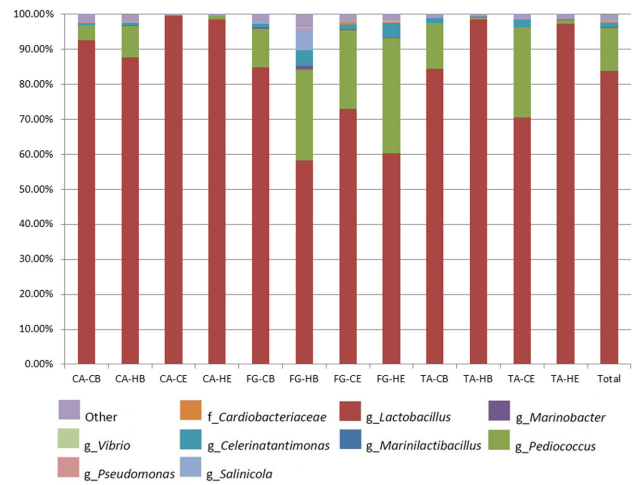

FIGURE 4 | Relative abundance of bacterial species in the different treatments assayed, obtained through metagenomics analysis of 16S RNA gene at the end of the fermentation process in brine (B) and epidermis of fruits (E). CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

on overall acceptability. At preselected sampling periods, the samples were offered to panelists, using blue glass according to the recommendations of the standard COI/T.20/Doc.No 5 (Glass for oil tasting) (International Olive Council [IOC], 1987), coded with three digits randomly chosen, and in a balance presentation with respect to PDO. All the attributes were evaluated on an unstructured scale which ranged from 1 to 11, in which 1 was associated with the complete absence of the attribute and 11 to its presence in the highest intensity. The panelists were asked to mark on the scale according to the intensity perceived of each attribute. The sheets were read by the panel leader with 0.1 cm precision.

Statistical Analysis

The data were subjected to an analysis of variance. For this purpose, the one-way ANOVA module of Statistica 7.1 software (Statsoft Inc., Tulsa, OK, United States) was used to check for significant differences among physicochemical, microbiological and sensory attributes as a function of the different treatments assayed. A *post hoc* comparison statistical LSD test was applied using $p \leq 0.05$ as the cut-off level of significance.

RESULTS

Physicochemical Changes during the Fermentation Process

Remarkable differences between heat-shocked and untreated olives were found for pH, titratable acidity, and salt content throughout the 138 days of fermentation (**Figure 1**). In CA treatments, pH increased from the initial 3.0 (first day after brining) up to 3.8 units at the end of the fermentation process. However, in FG treatments and the control following the traditional process (TA-C), a pH value close to 4.3 units was noticed during the entire fermentation time. An entirely different behavior was detected in TA-H, whose pH decreased

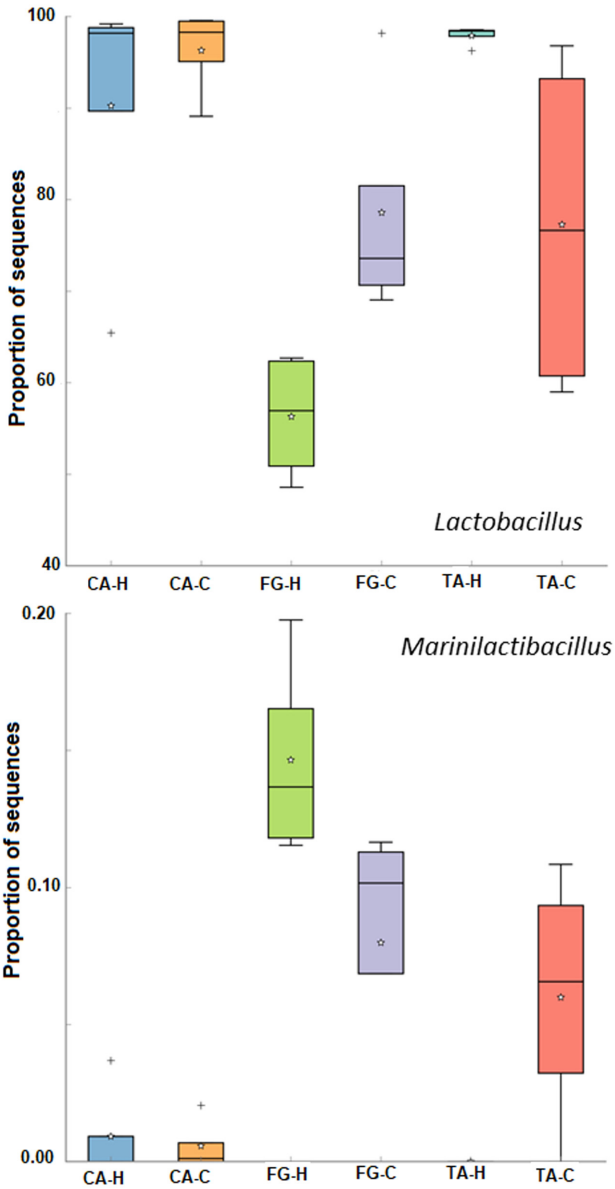

FIGURE 5 | ANOVA Tukey–Kramer *post hoc* comparison test executed with STAMP v2.0.1 to elucidate significant differents for *Lactobacillus* (upper panel) and *Marinilactibacillus* (lower panel) genera in the diverse treatments. Significant differences were only considered for $p \leq 0.05$ and a q-value below 0.3. Meaning of symbols: Cross, star and horizontal line inside box denoted for outlier, mean, and median of the percentage of sequences obtained. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

from an initial 4.3 value to a final 3.9 units at the end of the process. Titratable acidity values were kept constant at approximately 0.4% in FG and TA-C treatments throughout the fermentation period but increased for CA olives and the TA-H treatment. Interestingly, the application of a mild-heat-shock treatment to the fruits favored a higher production of titratable acidity in CA and TA treatments than in their respective controls. The evolution of salt in CA and TA/FG was also completely different, with a lower content in the equilibrium (∼4.5% NaCl) in CA than in TA/FG (∼9.0% NaCl) treatments.

The color data also revealed considerable differences among the diverse *Aloreña de Málaga* denominations (**Figure 2**). The loss in greenness was faster for CA fruits, followed by TA and FG olives. The maximum a^* value, which is associated with the worst green color, was observed in the CA treatments (∼7.5), followed by the control of TA (∼4.5) and FG (∼2.0). Notice the close position of TA-H treatment to FG at the end of the fermentation process (without significant differences between them at $p \leq 0.05$). A similar trend was followed by h_{ab}, although reversed. The lowest value was found in CA treatments (∼78°), followed again by TA-C (∼82°) and FG samples (∼87°). The position of TA-H fruits was again close to FG treatments (∼87°).

At the end of the fermentation process, the texture of CA treatments (which use whole fruits) was higher compared to the cracked olives used for the elaboration of TA and FG olives (**Table 2**). The total sugar content in brine was statistically different ($p \leq 0.05$) in the three *Aloreña de Málaga* commercial denominations. Sugars were practically exhausted in CA treatments but not in TA or, particularly, in FG. The acetic and lactic acid contents were higher ($p \leq 0.05$) in CA and TA-H than in the other treatments. However, the ethanol concentration showed the opposite behavior. The highest values ($p \leq 0.05$) were noticed in TA-C and FG.

Microbiological Changes during the Fermentation Process

Enterobacteriaceae were never found in any treatment. On the contrary, high population levels of yeasts (5.0–6.0 $\log_{10}$ CFU/mL) were always observed. This microbial group first appeared in TA (in both control and heat-treated fruits), then in CA and finally in FG (**Figure 3**, upper panel). Regarding the lactic acid bacteria (LAB) population, this gram-positive bacteria group was only detected in CA and TA-H treatments. The LAB were first noticed in TA-H (from 2 weeks onward) reaching population levels of approximately 5.5 $\log_{10}$ CFU/mL at the 50th day of fermentation. LAB appear later in the CA-H (olives subjected to the heat-shock treatment), with an approximate delay of 3 weeks, and finally in CA-C after 7 weeks of fermentation. In both CA denominations, the LAB population reached levels close to 7.0 $\log_{10}$ CFU/mL (**Figure 3**, lower panel). Except for FG, the heat-shock treatment stimulated the early presence of LAB and their growth. At the end of the fermentation process, the highest count ($p \leq 0.05$) was obtained in the CA treatment, followed by CA-H, and finally the TA-H treatment.

TABLE 4 | Physicochemical and microbiological data obtained for the diverse treatments and packaging storage periods.

		Physicochemical parameters						Microbiological parameters	
Treatment	**Packaging (days)**	**pH**	**Salt (%)**	**Titratable acidity (%)**	**Texture (kN/100 g)**	**Color a^***	**Color h_{ab}**	**LAB ($\log_{10}$ CFU/mL)**	**Yeast ($\log_{10}$ CFU/mL)**
CA-C	4	3.73 (0.01)^{d}	3.70 (0.01)^{b}	0.76 (0.02)^{f}	8.39 (1.52)^{a}	7.28 (0.09)^{e}	75.23 (0.15)^{c,d}	7.03 (0.03)^{a,b,c}	5.18 (0.07)^{c,d}
	41	3.82 (0.01)^{g}	3.81 (0.02)^{d}	1.16 (0.02)^{h}	7.27 (1.41)^{a}	7.43 (0.24)^{e}	74.37 (0.50)^{c}	7.55 (0.05)^{a}	4.34 (0.70)^{b}
CA-H	4	3.65 (0.02)^{a}	3.74 (0.01)^{c}	0.80 (0.03)^{g}	7.14 (0.98)^{a}	6.99 (0.07)^{e}	76.00 (0.68)^{d}	6.58 (0.43)^{b}	4.57 (0.08)^{b,c}
	41	3.59 (0.01)^{c}	3.70 (0.01)^{b}	1.26 (0.03)^{i}	6.48 (1.07)^{a}	6.15 (0.12)^{d}	78.39 (0.07)^{g}	6.73 (0.09)^{b,c}	3.60 (0.65)^{a}
FG-C	4	3.87 (0.08)^{h}	4.19 (0.01)^{e}	0.39 (0.02)^{a}	8.65 (1.53)^{a}	2.22 (0.41)^{a}	86.02 (0.73)^{a,b}	0.00 (0.00)^{f}	4.17 (0.07)^{a,b}
	41	4.04 (0.01)^{b}	4.52 (0.02)^{a}	0.41 (0.01)^{a}	8.92 (1.39)^{a}	3.40 (0.07)^{b}	84.08 (0.24)^{f}	5.03 (0.45)^{d,e}	3.64 (0.25)^{a}
FG-H	4	3.91 (0.01)^{i}	4.56 (0.01)^{a}	0.34 (0.01)^{b}	7.21 (1.54)^{a}	2.56 (0.06)^{a}	85.65 (0.02)^{a}	4.38 (0.46)^{d}	5.20 (0.03)^{c,d}
	41	4.00 (0.02)^{j}	4.43 (0.04)^{f}	0.50 (0.02)^{e}	6.21 (2.02)^{a}	3.28 (0.27)^{b}	84.27 (0.31)^{f}	7.65 (0.75)^{a}	4.17 (0.04)^{a,b}
TA-C	4	3.79 (0.01)^{f}	4.65 (0.01)^{g}	0.31 (0.04)^{c}	7.43 (1.71)^{a}	4.79 (0.08)^{c}	80.94 (0.06)^{e}	4.33 (0.22)^{d}	6.12 (0.02)^{e}
	41	4.03 (0.02)^{b}	4.53 (0.02)^{a}	0.38 (0.01)^{a}	7.19 (1.58)^{a}	4.52 (0.33)^{c}	80.82 (0.37)^{e}	7.17 (0.27)^{a,b,c}	4.05 (0.06)^{a,b}
TA-H	4	3.65 (0.01)^{a}	4.56 (0.01)^{a}	0.35 (0.01)^{b}	6.95 (2.19)^{a}	2.65 (0.31)^{a}	85.93 (0.52)^{a,b}	5.13 (0.03)^{e}	5.51 (0.12)^{d,e}
	41	3.75 (0.02)^{e}	4.83 (0.01)^{h}	0.45 (0.02)^{d}	6.70 (1.61)^{a}	2.11 (0.47)^{a}	86.78 (0.72)^{b}	7.41 (0.21)^{a,c}	4.15 (0.37)^{a,b}

%, expressed as w/v; standard deviation obtained from duplicate measurements in parentheses. Values followed by different superscript letters, within the same column, are statistically different ($p \leq 0.05$) according to the LSD posthoc comparison test. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to Aloreña de Malaga cultivar.

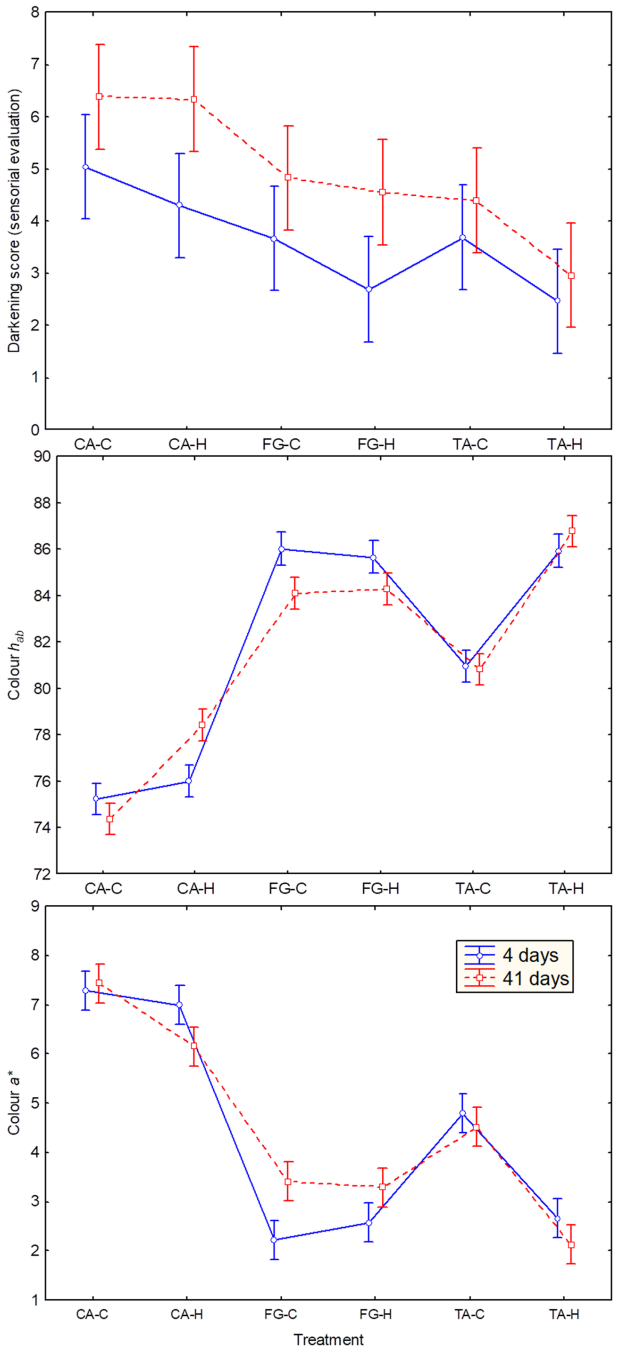

FIGURE 6 | Darkening score assigned by panelist **(upper)**, hue angle (h_{ab}, **middle**), and greenness (a^*, **lower**) concerning the diverse treatments. Error bars for instrumental measurements denote standard deviation calculated from duplicate packaging. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar

Metagenomic Analysis

A total of 945,386 raw sequences were obtained from the 24 olive samples analyzed in this work. After screening the data for poor quality sequences, the removal of chloroplasts and taxonomically unassigned 16S sequences, 307,772 sequences (an average of 25,647 sequences per sample) were finally used for the metagenomic analysis. Overall, despite the diversity in sequencing depth among samples (**Table 3**), the rarefaction analysis indicated that some reads above 6,583 per sample were satisfactory to obtain good coverage (always above 96%).

Table 3 shows the total of OTUs found in the different samples and their alpha-diversity indexes. In general, a higher biodiversity was noticed for FG *Aloreña* samples, which showed the highest values for Simpson and Shannon indexes. The total number of OTUs assigned ranged from 158 to 302, with an average of 204 observed OTUs per sample. The bacterial phylogenetic assignation of all samples showed that two bacterial phyla (*Proteobacteria* and *Firmicutes*) included the genera with the greatest number of sequences (**Figure 4**). The *Proteobacteria* represented only 2.4% of the total sequences, with genera *Celerinatantimonas* (1.32%), *Salinicola* (0.70%), *Marinobacter* (0.17%), *Pseudomonas* (0.08%), and *Vibrio* (0.06%) as the most representative. They were found in practically all samples. On the contrary, the phyla with the major number of sequences were *Firmicutes* (96.02% of total sequences), with genera *Lactobacillus* (83.67%), *Pediococcus* (12.30%), and *Marinilactibacillus* (0.05%) as the most abundant. **Figure 4** shows the relative abundance of bacterial genera for the different treatments assayed, making a distinction between samples obtained from brine (B) or fruit epidermis (E). The abundance of *Lactobacillus* in all FG samples and the TA-C treatment was the lowest, as confirmed by the application of the Tukey-Kramer *post hoc* test (**Figure 5**, upper panel). The proportion of sequences obtained for *Lactobacillus* genera, regardless of the origin (brine or fruit) was statistically lower ($p \leq 0.05$) in FG-H (56.28%), TA-C (77.27%), and FG-C (78.58%) than in CA-H (90.25%), CA-C (96.30%) and TA-H (97.90%) treatments. On the contrary, the presence of *Marinilactibacillus* genera was statistically higher ($p \leq 0.05$) in FG (0.15 and 0.08% for FG-H and FG-C, respectively) and TA-C samples (0.06%) than in the rest of the samples (which were below 0.01%); that is, this genera showed an opposite behavior compared to *Lactobacillus* (**Figure 5**, lowest panel).

Evaluation of Packaged Fruits

After the fermentation process, the fruits were packaged and subjected to physicochemical and microbiological analyses on the 4th and 41st day of storage (**Table 4**). *Enterobacteriaceae* were never detected in any packaging sample. On the contrary, high populations of LAB and yeasts were found. An increase in LAB population throughout packaging was noticed in practically all treatments while yeast counts had a statistically significant reduction ($p \leq 0.05$), except in the FG-C treatment, during the same period. Concerning physicochemical data, pH ranged from 3.59 (CA-H) to 4.04 (FG-C) at the 41st day of packaging, with a slight trend to increase as the packaging time progressed. After the same period, the salt content ranged from 3.70 (CA-H) to 4.93% (TA-H), with lower values for the CA treatments. Titratable acidity statistically increased ($p \leq 0.05$), from 0.34 (FG-H) on the 4th day to 1.26% (CA-H) on the 41st day of packaging, due to the simultaneous increment in the LAB population.

TABLE 5 | Scores assigned by the panelist to the sensory attributes of the diverse treatments according to packaged storage periods.

Treatment	Packaging storage (*d*)	Hardness	Crunchiness	Acidic	Salty	Bitterness	Browning	Defects	Overall acceptability
CA-C	4	7.60 (2.25)^{a}	7.57 (2.16)^{a}	6.47 (2.65)^{c,d}	4.37 (1.77)^{a,b,c,d}	4.21 (2.10)^{a,c}	5.03 (2.47)^{a,e}	3.88 (2.98)^{a}	6.58 (2.18)^{a,b,c}
	41	7.63 (2.09)^{a}	7.87 (1.50)^{a}	7.07 (2.12)^{c,d}	5.50 (1.47)^{b}	5.75 (2.58)^{b}	6.37 (2.74)^{e}	5.17 (2.87)^{a}	5.42 (1.56)^{b,d}
CA-H	4	7.91 (1.89)^{a}	7.71 (1.67)^{a}	6.01 (2.30)^{c,e}	4.90 (1.67)^{a,b,c,d}	3.60 (1.40)^{a}	4.30 (2.70)^{a,c}	3.13 (1.74)^{a}	7.51 (1.38)^{a}
	41	6.50 (2.42)^{a}	6.48 (2.13)^{a}	7.24 (1.75)^{c,d}	5.15 (1.71)^{b,c,d}	5.22 (2.36)^{b,c}	6.32 (1.45)^{e}	4.54 (2.58)^{a}	5.52 (1.97)^{b,c,d}
FG-C	4	7.86 (1.83)^{a}	8.33 (1.40)^{a}	4.23 (1.88)^{a,b}	3.91 (1.63)^{a,c}	4.92 (2.34)^{a,b,c}	3.66 (2.09)^{a,b,c}	4.10 (2.36)^{a}	6.74 (1.56)^{a,c}
	41	7.77 (1.79)^{a}	7.66 (1.77)^{a}	5.19 (2.12)^{a,b,c,e}	4.34 (1.84)^{a,b,c,d}	4.77 (1.83)^{a,b,c}	4.82 (1.87)^{a}	3.64 (1.90)^{a}	6.55 (1.30)^{a,b,c}
FG-H	4	6.70 (2.63)^{a}	6.91 (2.23)^{a}	3.85 (1.77)^{a}	3.64 (1.22)^{a}	3.57 (1.44)^{a}	2.68 (1.35)^{b}	3.93 (2.03)^{a}	6.88 (1.39)^{a}
	41	6.22 (2.47)^{a}	6.28 (2.38)^{a}	7.54 (2.51)^{d}	5.61 (2.69)^{b}	7.41 (1.98)^{d}	4.54 (1.82)^{a}	5.03 (2.83)^{a}	3.97 (1.86)^{e}
TA-C	4	7.25 (2.21)^{a}	7.42 (2.03)^{a}	4.50 (1.33)^{a,b,e}	3.76 (1.21)^{a}	3.61 (1.55)^{a}	3.68 (0.68)^{a,b,c}	3.85 (2.50)^{a}	6.36 (2.53)^{a,b,c,d}
	41	7.45 (1.46)^{a}	6.93 (1.99)^{a}	6.30 (2.10)^{c,d}	4.82 (2.11)^{a,b,c,d}	5.66 (2.10)^{b,c}	4.39 (2.04)^{a}	4.84 (2.22)^{a}	5.17 (1.49)^{d,e}
TA-H	4	7.09 (1.81)^{a}	6.68 (1.53)^{a}	4.44 (1.42)^{a,b}	4.14 (1.69)^{a,c,d}	3.50 (1.43)^{a}	2.46 (0.83)^{b}	3.79 (2.20)^{a}	6.87 (1.34)^{a}
	41	6.50 (1.79)^{a}	6.68 (1.53)^{a}	5.55 (1.87)^{b,c,e}	5.45 (1.76)^{b,d}	5.73 (2.09)^{b}	2.95 (1.36)^{b,c}	3.24 (1.54)^{a}	6.90 (2.03)^{a}

Standard deviation in parentheses (n = 14). Values followed by different superscript letters within the same column are statistically different ($p \leq 0.05$) according to the LSD post hoc comparison test. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the Aloreña de Malaga cultivar.

The instrumental texture between heat-shocked fruits and their respective controls was not statistically significant ($p \geq 0.05$). The major effects were noticed on the fruits' color expressed as greenness (a^*) and hue angle (h_{ab}). The best color appearance of the fruits was obtained for chilled olives (FG) as well as for the traditional process using heat-shocked fruits (TA-H), which showed significant differences (at $p \leq 0.05$) with respect to the other treatments. On the contrary, the worst instrumental color values were noticed for CA olives. Also, there was a significant ($p \leq 0.05$) loss in color throughout the shelf life in most of the treatments, except in TA-H (**Figure 6**).

With regards to the sensory evaluation (**Table 5**), there were no significant differences ($p \leq 0.05$) among treatments and packaging days for hardness, crunchiness or defects. The first two attributes always obtained good scores (>6.2) while they were lower for the latter (only two treatments exceed 5.0 at the end of packaging). There were significant differences ($p \leq 0.05$) in acidic, salty and bitter among the three *Aloreña de Malaga* denominations and between packaging times but not between heat-shocked olives and their respective controls. Furthermore, acidic, salty and bitterness usually increased in all treatments from the 4th to 41st days. Important browning differences among treatments were detected by panelists, with the highest brown values assigned to CA olives at the end of the storage period (6.3). On the contrary, the lowest values were obtained by TA-H (2.4). In general, browning scores increased as time progressed with statistically significant differences ($p \leq 0.05$) for CA-H and FG-H (**Figure 6**, upper panel). Finally, the overall acceptability score at the beginning of packaging was generally high (>6.5) but decreased considerably in some treatments after 41 days (CA-C, CA-H, FG-H, TA-C), except FG-C and TA-H which kept their high scores throughout the packaging period.

DISCUSSION

Etchells et al. (1964) used hot-water blanching (66–80°C) for a short time (5 min) to rid cucumbers of naturally occurring, interfering, and competitive microbial groups before brining. Inoculation with the desired LAB of the treated material led to the pure culture fermentation of brined cucumbers. The application of a similar treatment to olives (74°C for 3 min) not only inhibited the initial wild microbiota but improved their fermentation (Etchells et al., 1966). The effect was linked to the presence of a LAB inhibitor in the fresh olives that, apparently, was degraded by the heat-shock (Fleming and Etchells, 1967). The use of hot-alkaline solutions improved the fermentation, with a marked enhancement of the acidification rates of *Merhavia* and *Manzanilla* green olives (Juven et al., 1968). Montedoro et al. (2002) was the first to link the lower concentration of HyEDA to a heat treatment of olives. An initial heat-shock treatment (80°C for 10 min) was also applied to reduce the wild microorganisms adhered to the olive epidermis and facilitate the brine and olive surface colonization by *Lactobacillus pentosus* B281 (Argyri et al., 2014). Recently, Ramírez et al. (2017) carried out a mild heat treatment (60°C, 10 min) of olives, followed by a direct brining and inoculation with selected LAB strains. The process caused

oleuropein depletion and reduced the natural bitterness of fruits without the application of any alkali hydrolysis. Apparently, the heat treatment inactivated the β-glucosidase activity of fruits and prevented the formation of antimicrobial compounds like HyEDA while promoting LAB growth.

After these research works, heat-shock should be considered as a promising treatment for LAB growth improvement in brined olives. Obviously, in the case of cultivars with low oleuropein content, such as *Aloreña de Málaga,* the benefits could be even greater. The results obtained in the present study for the cured and traditional denominations have confirmed this hypothesis since a strong LAB growth was observed in CA-H and TA-H denominations (**Figure 3**), which can be linked to the inactivation of the β-glucosidase enzyme and the subsequent absence of HyEDA. However, in not heat treated olives, the formation of inhibitors, although in a limited proportion, was enough to cause a moderate LAB population reduction. This is in agreement with the observations reported by Medina et al. (2007), who found the inhibition of LAB growth even at 0.25 mM concentrations of HyEDA during the storage of natural green olives in brine without alkali treatment. However, the results obtained in this work also indicate an inhibition of the β-glucosidase by temperature (Ramírez et al., 2014) as a consequence of the adequate selection of the heat treatment (60°) which took advantage of the drastic decrease in the activity of this enzyme above 50°C but was good enough to preserve texture, a highly appreciated attribute in *Aloreña de Malaga* olives.

The heat-shock treatment also had a marked effect on the microbiota. In this work, the microbial populations of the olives which received a heat treatment consisted mainly of *Lactobacillus* and *Pediococcus*. In contrast, Medina et al. (2016), using pyrosequencing analysis, reported the presence of undesirable *Celerinatantimonas, Pseudomonas,* and *Propionibacterium* as the most abundant genera detected in traditional industrially fermented fruits while the species of the *Lactobacillaceae* family were in low proportion (3–8%). This work also reveals information about the bacterial biodiversity for CA and FG *Aloreña de Málaga* denominations, whose alpha-biodiversity indexes and number of OTUs obtained in the present work were considerably higher than in previous studies (Medina et al., 2016).

The only disadvantage to exposing olives to a heat treatment could be firmness and color deterioration, with the subsequent impact on consumer acceptance (Brenes et al., 1994). However, no significant effect on olive firmness was found in this work, and the results that are in agreement with those reported by Ramírez et al. (2017). Interestingly, the color of the heat-shocked olives was better than the controls which were browner and had higher a^* values. According to Ramírez et al. (2017), these effects could have been due to the inactivation of another enzyme, the polyphenol-oxidase (PPO), by the heat-shock treatment with the subsequent delay in phenolic compound oxidation, polymerisation, and olive darkening.

In summary, the application of a mild heat-shock to *Aloreña de Málaga* fruits was beneficial, especially for the traditional process, since it favored the growth of the LAB population (especially *Lactobacillus* genera), caused a higher retention of the green appearance, and improved the stability of the packaged olives. Furthermore, all these changes occurred without any adverse effects on the sensory characteristics of the packaged products.

AUTHOR CONTRIBUTIONS

FR-G, MR-B, and VR-G performed the experimental work. AB-C executed the metagenomics analysis. FR-G and FA-L designed the work, while FA-L and AG-F analyzed the results and wrote the paper.

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3.2 SECCIÓN II

3.2.1 CAPÍTULO 6

A probabilistic decision-making scoring system for quality and safety management in Aloreña de Malaga table olive processing

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ORIGINAL RESEARCH
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A Probabilistic Decision-Making Scoring System for Quality and Safety Management in *Aloreña de Málaga* Table Olive Processing

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Table olives are one of the most representatives and consumed fermented vegetables in Mediterranean countries. However, there is an evident lack of standardization of production processes and HACCP systems thus implying the need of establishing decision-making tools allowing their commercialization and shelf-life extension. The present work aims at developing a decision-making scoring system by means of a probabilistic assessment to standardize production process of *Aloreña de Málaga* table olives based on the identification of potential hazards or deficiencies in hygienic processes for the subsequent implementation of corrective measures. A total of 658 microbiological and physico-chemical data were collected over three consecutive olive campaigns (2014–2016) to measure the variability and relative importance of each elaboration step on total hygienic quality and product safety. Three representative companies were visited to collect samples from food-contact surfaces, olive fruits, brines, air environment, olive dressings, water tanks, and finished/packaged products. A probabilistic assessment was done based on the establishment of Performance Hygiene and Safety Scores (PHSS 0–100%) through a standardized system for evaluating product acceptability. The mean value of the global PHSS for the *Aloreña de Málaga* table olives processing ($PHHS_{FTOT}$) was 64.82% (90th CI: 52.78–76.39%) indicating the high variability among facilities in the evaluated processing steps on final product quality and safety. Washing and cracking, and selection and addition of olive dressings were detected as the most deficient ones in relation to $PHSS_{Fi}$ values ($p < 0.05$) (mean = 53.02 and 56.62%, respectively). The relative contribution of each processing step was quantified by different experts ($n = 25$) from the *Aloreña de Málaga* table olive sector through a weighted PHSS ($PHSS_W$). The mean value of $PHSS_W$ was 65.53% (90th CI: 53.12–77.52%). The final processing steps obtained higher values for $PHSS_W$ being the finished product the most relevant one (mean = 18.44%; 90% CI: 10.34–25.33%). Sensitivity

analysis concluded that intervention measures focused on reducing the contamination of washing brines could lead to an improvement of $PHSS_{FTOT}$ value to 67.03%. The present work can be potentially applied in the *Aloreña de Málaga* table olive food sector for improving food quality and safety assurance.

Keywords: table olives, HACCP, decision-support system, performance hygiene and safety scores, sensitivity analysis

INTRODUCTION

Table olives are one of the most representative and consumed fermented vegetables in Mediterranean countries (Garrido-Fernandez et al., 1997; Arroyo-López et al., 2012, 2016). According to the recent statistics provided by the International Olive Oil Council (IOOC), European production has raised in 2015/16 to 859.8 mT whereas consumption also showed an increasing trend to 410.7 mT (IOOC, 2017). The global consumption of table olives in recent years has multiplied by 2.7, increasing by 182.0% over the period 1990/91–2016/17. Spain was ranked as the main producer in the world as well as the main consumer with 4.1 kg/person/year.

In the last years, consumers are demanding healthier and more convenient table olive preparations based on traditional processes. In Spain, *Aloreña de Málaga* green table olive has a Protected Designation of Origin (PDO) due to their peculiar characteristics of elaboration and geographical production region (Guadalhorce Valley, Málaga, Spain). Due to its low-to-moderate concentrations of oleuropein, the processing does not include alkaline debittering. Thus, they are produced as directly brined cracked green olives and seasoned with diverse herbs and species before packaging (López-López and Garrido-Fernández, 2006). Their differential characteristics regarding other table olive varieties limits the possibility of applying a heat treatment sufficiently high to destroy or reduce the microbial load in the packaged product. This requires the implementation of alternative preservation processes to allow increasing the shelf-life and further commercialization of finished products.

The microbiological safety of foods is managed by the effective implementation of control measures within a Food Quality Safety Management Systems (FQSMS) including prerequisite programme (PRP) and hazard analysis and critical control points (HACCP) that have been validated, where appropriate, throughout the food chain to minimize contamination and improve food safety (Valero et al., 2017). An integrated approach to food safety covers all sectors of the food chain (Regulation EC 178/2002, Commission Regulation, 2002) in response to requirements demanded by customers, competent authorities and certification bodies. Hygienic requirements for foodstuffs (Regulation EC 852/2004, Commission Regulation, 2004) implemented in the EU have urged the need to develop more sophisticated food quality and safety assurance standards and guidelines (Tzamalis et al., 2016). For the table olives sector, the codex standard (CODEX STAN 66-1981, review 1987 and 2013, Codex Alimentarius Commission, 1981) and the Trade Standard Applying to Table Olives (IOC, 2004) recommend that the product covered by these documents must be prepared and handled in accordance with the appropriate sections of the General Principles of Food Hygiene (CAC/RCP 1-1969; Codex Alimentarius Commission, 1969b), the Code of Hygienic Practice for Low-Acid and Acidified Low-Acid Canned Foods (CAC/RCP 23-1979, Codex Alimentarius Commission, 1969a, 1979), and the Code of Hygienic Practice for Canned Fruit and Vegetable Products (CAC/RCP 2-1969, Codex Alimentarius Commission, 1969a). In addition, the product should comply with any microbiological criteria established in accordance with the Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21-1997, Codex Alimentarius Commission, 1997; Regulation (EC) 1441/2007, Commission Regulation, 2007).

The development of a FQSMS requires quantitative tools able to assess the acceptance of final products. However, there is an evident lack of standardization in the table olive sector, thus implying the need of establishing decision-making tools allowing their commercialization and shelf-life extension. Lack of experience, knowledge and human and financial resources make difficult the implementation of standardized FQSMS in industry (Tzamalis et al., 2016). Further, production of table olives as fermented products could not be standardized since several factors such as variations in olive composition according to the season, spontaneous fermentation processes, limited technological capabilities in the company or lack of scientific and technical knowledge by industry's operators. Specifically, the manufacturing process of *Aloreña de Málaga* table olives is carried out by small and medium enterprises placed in, or very close to, the region of production. This fact together with the limited shelf-life of final products due to the presence of high residual sugars, spoilage microorganism, clouding or brines and swelling containers, make the distribution area very limited and do not allow in some cases exportation to other countries (Romero-Gil et al., 2016). There are previous studies dealing with the development of FQSMS in other food commodities demonstrating their usefulness to improve food quality and safety. One of the best examples is the Food Safety Management System- diagnostic instrument (FSMS-DI), which contributes to the measurement of the performance of the FSMS in an organization suggested for edible oil or fresh produce chains (Nanyunja et al., 2015; Ren et al., 2016). Further, development of scoring systems (Stadlmüller et al., 2017) and best practice scores (Tzamalis et al., 2016) for the assessment of FQSMS are also reported. However, these systems are deterministic approaches mainly based on performance of questionnaires or microbial data on targeted hazards, being not potentially applied to the table olive sector. The establishment of risk quality or safety margins by food operators is desirable since quantification of

variability associated to products and processes can be quantified. Furthermore, these measures are in line with the preventive Food Safety Modernization Act approach implemented in US (Grover et al., 2016).

In this study, a probabilistic approach is suggested to assess the performance of quality and safety of *Aloreña de Málaga* table olives production. Based on physico-chemical and microbiological data collected from three representative companies and seasons in Southern Spain, a decision-scoring system was developed establishing Performance Hygiene and Safety Scores (PHSS) to identify potential factors and processing steps to operationalize hygiene and safety of table olive processing.

MATERIALS AND METHODS

Study Design and Facilities

This study was performed in three different small and medium companies dedicated to table olives production located in Southern Spain (Valle del Guadalhorce, Málaga, Spain) which process all *Aloreña de Málaga* table olives. The experimental work was conducted in three consecutive campaigns from 2014 to 2016. Types of samples, processing steps to analyse and sampling planning were previously agreed with the quality inspector of each company as indicated in their different industry's Self-Control Plan (HACCP). The processing steps considered for the present study were based on the traditional elaboration of *Aloreña* table olives and they are shown in **Figure 1**. From each step, different microbiological and physico-chemical analyses were performed as described below to determine their influence on final product quality, hygiene and safety.

FIGURE 1 | Flow diagram of the elaboration process of *Aloreña* table olives shared by the different industries visited in the study.

Microbiological Analyses

Samples Collection

According with the sampling planning (**Tables 1**–**5**), different types of samples were collected in the industry, transferred to sterile containers, transported to the laboratory at refrigeration (2–4°C) conditions and analyzed within 24 h after collection.

Enumeration of Microbial Populations in the Different Types of Samples

To enumerate microbial populations in brines, samples (10 ml), if necessary, were serially diluted in sterile saline solution (0.9% NaCl) and plated (50 μl) in the correspondent culture media described below through using a Spiral Plater model dwScientific (Don Whitley Scientific Limited, UK). After incubation periods at the different temperatures according to the microbial group analyzed in this type of sample [lactic acid bacteria (LAB), yeasts and molds (Y/M), mesophilic bacteria (MB), and *Enterobacteriaceae* (Ent)], colonies were counted by using an Image Analysis System model CounterMat v.3.10 (IUL, Barcelona, Spain). Results were expressed at $\log_{10}$ cfu/ml.

For determination of microorganisms present in olive fruits [MB, Y/M, LAB, Ent, coagulase positive *Staphylococci* (CPS), sulphite reducing clostridia (SRC), *Listeria monocytogenes* (LM), and *Salmonella* sp. (Salm)], two olives (approximately 10 g) were washed with sterile saline solution (0.85% v/v) and deboned at sterile conditions. Then, a decimal dilution of fruit flesh in saline solution (90 ml) was homogenized in a Stomacher 400 Circulator Blender (Seward Laboratory System, UK) for 5 min. Afterwards, 50 μl were plated in the selective culture media. Results were expressed at $\log_{10}$ cfu/g.

To enumerate the number of microorganisms present in olive dressings (MB, Y/M, LAB, Ent, CPS, and SRC), 10 g of each seasoning material (garlic, red pepper and herbal mixture) were singly homogenized for 5 min in Stomacher with 90 ml of buffered peptone water (0.1%). Afterwards, 50 μl of the solutions were plated in the different culture media. Results were expressed at $\log_{10}$ cfu/g.

To determine the presence of microorganisms in water, samples (1,000 ml) were poured into sterile flasks and re-suspended with 5% solution of sodium thiosulfate (Panreac, Barcelona, Spain) to remove the residual effect of free chlorine. Then, samples were filter-sterilized using 0.22 μm diameter filters (Merck Millipore, Massachusetts, US). Then, filters were transferred to different selective media for analysis of the microbial groups specified in the Spanish Royal Decree (RD 140/2003, Royal Decree, 2003) such as MB, coliforms (Col), and SRC. Results were expressed at $\log_{10}$ cfu/ml.

TABLE 1 | Analyses performed, parameters, concentration and scores obtained from samples collected at processing step No. 1 (reception of raw materials and fermentation).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	208.87 (181.44, 236.31)	1.56 (1.95, 2.30)
		Y/M	56.50 (24.98, 88.02)	1.00 (0.74, 1.26)
Olive brine (32)	log_{10} cfu/ml	MB	6.20 (5.91, 6.37)	2.25 (1.77, 2.73)
		Y/M	5.00 (4.61, 5.21)	1.75 (1.35, 2.15)
		LAB	6.30 (5.98, 6.48)	2.00 (1.55, 2.45)
		Ent	<1.30 (–)	0.00 (–)**
Olive fruit (48)	log_{10} cfu/g	MB	5.88 (5.54, 6.06)	1.75 (1.42, 2.08)
		Y/M	4.30 (4.04, 4.47)	1.25 (0.96, 1.54)
		LAB	5.79 (5.48, 5.97)	1.50 (1.16, 1.84)
		Ent	<1.30 (–)	0.00 (–)
		CPS	<1 (–)	0.00 (–)
		SRC	1.07 (1.01, 1.11)	0.50 (0.20, 0.80)
Olive brine (12)	–	pH	4.23 (4.04, 4.42)	1.50 (0.52, 2.48)
	g/100 ml	FA	0.77 (0.62, 0.92)	0.00 (–)
	% (w/v)	NaCl	7.44 (7.10, 7.77)	0.00 (–)
Water (18)	cfu/100 ml	MB	14.30 (<10, 23.96)	0.83 (0.64, 1.02)
		Col	<10 (–)	1.00 (0.28, 1.72)
		SRC	<10 (–)	1.00 (0.28, 1.72)

MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; FA, free acidity; Col, total coliforms.

***CI 95% could not be estimated.*

Microbial air quality was determined by using an Air Sampler (SAS Super 180TM, Scharlab, Barcelona, Spain) searching for MB and Y/M as microbial indicators. The volume of air was fixed at 500 litters. Probable counts (*Pr*, cfu/m^3, statistical probability of multiple particles passing through the same hole) were obtained using a conversion table provided by the manufacturer.

The analysis of food-contact surfaces was carried out using MB and Ent as microbial indicators. Sterile polypropylene swabs (Nuovo Aptaca, Canelli, Italy) with amies medium were used for surface sampling. Each surface was swabbed using a 10 × 10 cm sterile metal template, then the swab head (1–2 cm) was aseptically cut and immersed in 3 ml test tubes of 0.1% buffered peptone water. In the case of handlers' gloves samples, the inner part was swabbed and the area in contact with hands was estimated as 225.07 ± 21.07 cm^2 for men and 188.03 ± 16.08 cm^2 for women (Ren et al., 2010). Results were expressed at cfu/cm^2.

Selective culture media used for enumeration of LAB was to DeMan Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) supplemented with 0.02% of sodium azide (Sigma, St. Louis, US) following by an incubation at 37°C for 48 h. Y/M were enumerated with the Yeast Mold agar (YM, Disco, Becton y Dickinson Company, Spark, MD, US) supplemented with 0.005% of gentamycin and oxy-tetracycline sulfate (Oxoid). Samples were incubated at 30°C for 48 h. Ent were counted using Violet Crystal Red Bile Dextrose (VRBD) agar (Merck, Darmstadt, Germany) after an incubation at 37°C for 24 h. MB were enumerated with Plate Count Agar (PCA, Oxoid) after an incubation at 28°C for 24h. CPS were enumerated following the ISO method (ISO: 6888-2: ISO, 1999) in Baird Parker supplemented with fibrinogen and rabbit plasmid (incubation at 37°C for 24 h). SRC were counted using Tryptose Sulphite Cycloserine (TSC) agar (Oxoid) after an incubation at 37°C for 24 h in anaerobic jars. Finally, presence of LM and Salm was confirmed using the ISO methods [ISO 11290-1/-2 (ISO, 2004, 2017) for LM and ISO 6579 (ISO, 2002) for Salm, respectively].

Physico-Chemical Analyses

The analyses of the olive brine for pH, salt, and titratable/free acidity (FA) were carried out using the routine methods described by Garrido-Fernandez et al. (1997). Total sugar content in brine (g/l) was determined by HPLC according to the methods developed by Sánchez et al. (2000) by the summation of values obtained for glucose, fructose, sucrose and mannitol.

Development of a Decision-Making Scoring System to Operationalize Hygiene and Safety of Table Olive Processing

Scoring System

In this study, a quantitative system assessing the food hygiene and safety throughout the elaboration process of table olives was established. This was done through a scoring system assigning different weighted values to microbiological and physico-chemical results obtained at the different steps in the elaboration chain (**Figure 1**). Scores ranged from 0 to 3, indicating the best and worst quality/safety conditions, respectively. Assigned scores and ranges are represented in **Table 6**. which describes how concentrations are translated to scores and the references used. These values were based on previous published studies, Codex standards for table olives, national and European legislations regarding different food criteria applied to samples

TABLE 2 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 2 (washing and cracking).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	210 (183.47, 236.77)	2.00 (–)
		Y/M	103 (79.83, 126.91)	1.38 (1.14, 1.61)
Hopper surface (16)	cfu/cm^2	MB	36.60 (5.44, 67.70)	2.63 (2.14, 3.00)
		Ent	73.5 (12.80, 134.17)	1.50 (0.76, 2.24)
Olive fruit (36)	log_{10} cfu/g	MB	5.70 (5.41, 5.87)	1.67 (1.22, 2.11)
		Y/M	4.66 (4.40, 4.82)	0.67 (0.33, 1.00)
		LAB	5.95 (5.76, 6.08)	1.67 (1.22, 2.11)
		Ent	<1.30 (–)**	0.00 (–)
		CPS	1.07 (<1, 1.11)	0.17 (0.03, 0.30)
		SRC	<1.30 (–)	0.00 (–)
Olive brine (24)	log_{10} cfu/ml	MB	5.04 (4.59, 5.25)	1.83 (1.37, 2.30)
		Y/M	4.87 (4.63, 5.03)	1.83 (1.37, 2.30)
		LAB	6.59 (6.03, 6.83)	1.33 (0.73, 1.94)
		Ent	4.38 (3.95, 4.59)	1.33 (0.79, 1.88)
Water (18)	cfu/100 ml	MB	1.34 (1.06, 1.50)	0.83 (0.64, 1.02)
		Col	<1.30 (–)	1.00 (0.28, 1.71)
		SRC	<1.30 (–)	1.00 (0.28, 1.71)

MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; Col, total coliforms.

***CI 95% could not be estimated.*

and parameters evaluated (Al Dagal et al., 1992; Federation des Industries Condimentaires de France, 2000; Royal Decree 1230/2001, Royal Decree, 2001; Royal Decree 140/2003, Royal Decree, 2003; IOC, 2004; Sneed et al., 2004; Regulation EC 1441/2007, Commission Regulation, 2007; Codex 66-1981, 2013, Codex Alimentarius Commission, 1981).

Calculation of Performance Hygiene and Safety Scores

The obtained results were processed and the correspondent scores assigned to each analytical data in accordance to the criteria represented in **Table 6**. Then, a probabilistic model was created in @Risk v7.5 (Palisade Corporation) to quantify variability associated to the elaboration process and to identify potential steps and factors that could influence on the final degree of hygiene and safety.

The variables used for model development were:

- F_i defining the processing step i (**Figure 1**) (i ranges from 1 to 5),
- T_i is the type of sample collected within the ith processing step (i.e., air environment, olive fruits, brines, etc.)
- P_i is the parameter analyzed corresponding to T_i within the processing step F_i (i.e., MB, LAB, pH, etc.) and,
- S_i is the assigned score to the ith parameter, ranging from 0 to 3.

As an example, for the processing step F_1 (reception of raw material and storage), and type of sample T_1 (air environment) the assigned scores as 0, 1, 2, and 3 were summed up for all parameters as follows:

$$(F_1, T_1) = \Sigma S_0(P_i);\ \Sigma S_1(P_i);\ \Sigma S_2(P_i);\ \Sigma S_3(P_i); \quad (1)$$

being P_i the number of parameters evaluated for this type of sample (in this case, MB and Y/M).

Let N_0 to N_3 be the number of times the scores were assigned as 0, 1, 2, and 3, then: $N_0 = \Sigma S_0; N_1 = \Sigma S_1; N_2 = \Sigma S_2; N_3 = \Sigma S_3$. Once N_0 to N_3 values were obtained, within each processing step (F) and type of sample (T), the correspondent probabilities (p) associated to each score were calculated as:

$$p_0 = \frac{N_0}{N_0 + N_1 + N_2 + N_3}; p_1 = \frac{N_1}{N_0 + N_1 + N_2 + N_3};$$
$$p_2 = \frac{N_2}{N_0 + N_1 + N_2 + N_3}; p_3 = \frac{N_3}{N_0 + N_1 + N_2 + N_3} \quad (2)$$

being $p_0 + p_1 + p_2 + p_3 = 1$. A discrete function was implemented in @Risk to assign any possible values from 0 to 3 as a function of the calculated probabilities (p). The resulting values from the discrete distribution (D_1, D_2 … D_i) corresponding to the types of samples and parameters evaluated were summed up to obtain the score for the ith processing step (D_{Fi}), defined as:

$$D_{Fi} = D_1T_1 + D_2T_2 + \ldots + D_iT_i \quad (3)$$

To measure the degree of fulfillment of the ith processing step on product quality and safety, a Performance Hygiene and Safety Score ($PHSS_{Fi}$, %) was obtained. $PHHS_{Fi}$ values ranged from 0 to 100% indicating the percentage of fulfillment of F_i on the overall quality and safety of the process and finished product and was calculated as follows:

$$PHSS_{Fi} = 1 - \left(\frac{D_{Fi}}{D_{maxFi}}\right) x\ 100 \quad (4)$$

TABLE 3 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 3 (selection and addition of olive dressings).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	182 (150.76, 213.80)	2.25 (2.02, 2.48)
		Y/M	81.4 (64.62, 98.12)	1.25 (1.02, 1.48)
Conveyor belt (12)	cfu/cm^2	MB	18,000 (0, 23,567)	3.00 (–)**
		Ent	94.2 (22.21, 166.08)	1.33 (0.60, 2.07)
Olive fruit (36)	log_{10} cfu/g	MB	5.19 (5.06, 5.30)	1.50 (1.23, 1.77)
		Y/M	4.07 (3.76, 4.25)	1.00 (0.71, 1.29)
		LAB	4.93 (4.70, 5.08)	1.33 (1.00, 1.67)
		Ent	2.30 (–)	0.50 (0.10, 0.90)
		CPS	<1.30 (–)	0.17 (0.03, 0.30)
		SRC	<1.30 (–)	0.00 (–)
Olive dressing: red pepper (36)	log_{10} cfu/g	MB	3.21 (3.09, 3.30)	0.33 (0.16, 0.50)
		Y/M	3.37 (2.93, 3.59)	0.50 (0.23, 0.77)
		LAB	2.72 (2.02, 2.98)	0.17 (0.03, 0.30)
		Ent	1.70 (1.00, 1.95)	0.50 (0.10, 0.90)
		CPS	2.90 (2.63, 3.07)	1.50 (0.96, 2.04)
		SRC	<1.30 (–)	0.00 (–)
Olive dressing: garlic (36)	log_{10} cfu/g	MB	4.61 (4.26, 4.79)	0.83 (0.40, 1.27)
		Y/M	3.13 (2.80, 3.31)	0.33 (0.16, 0.50)
		LAB	3.80 (3.50, 3.98)	0.83 (0.51, 1.15)
		Ent	3.15 (2.60, 3.38)	1.00 (0.49, 1.50)
		CPS	2.50 (2.16, 2.69)	1.00 (0.49, 1.50)
		SRC	<1.30 (–)	0.00 (–)
Olive dressing: herbs (36)	log_{10} cfu/g	MB	7.34 (6.69, 7.59)	2.50 (2.23, 2.77)
		Y/M	5.72 (5.43, 5.90)	1.67 (1.27, 2.06)
		LAB	5.56 (5.09, 5.78)	1.00 (0.49, 1.50)
		Ent	6.45 (5.75, 6.71)	2.00 (1.50, 2.50)
		CPS	4.42 (3.85, 4.66)	2.50 (2.10, 2.90)
		SRC	2.03 (1.74, 2.21)	1.00 (0.71, 1.29)
Handlers' gloves (12)	cfu/cm^2	MB	2.06 (0.80, 2.35)	1.60 (1.09, 2.11)
		Ent	<1 (–)	0.00 (–)

**MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; FA, free acidity; Col, total coliforms.*

***CI 95% could not be estimated.*

where D_{maxFi} was defined as the maximum score that can be potentially obtained for the processing step F_i (being representative of the worst-case scenario):

$$D_{maxFi} = 3 \times \text{number of parameters evaluated in } F_i.$$

This worst-case scenario was considered as the score 3 was associated to the poorest hygienic conditions. This measure was needed for model development to relativize the PHSS within each processing step.

Finally, the different scores obtained for the five processing steps were then summed up and a global score was obtained (D_{TOT}):

$$D_{TOT} = D_{F1} + D_{F2} + D_{F3} + D_{F4} + D_{F5} \quad (5)$$

With this information, the global Performance Hygiene and Safety Score ($PHSS_{FTOT}$, %) was calculated as:

$$PHSS_{F_{TOT}} = 1 - \left(\frac{D_{TOT}}{D_{\max F_{TOT}}} \right) x\, 100 \quad (6)$$

where $D_{maxF_{TOT}}$ was defined as the maximum score that can be potentially obtained for the five processing steps evaluated.

Calculation of Weighted Performance Hygiene and Safety Scores ($PHSS_w$)

To measure the relative importance of each processing step on the final quality and safety of table olives, an Expert Knowledge Elicitation process (EKE) was performed. Expert elicitation is a process for quantifying expert opinion regarding uncertainties to address research problems in areas where traditional scientific research is infeasible or not yet available. Because uncertainties in the probabilistic model can be described in terms of probability

TABLE 4 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 4 (packaging processes).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	184 (150.46, 216.73)	2.17 (1.97, 2.37)
		Y/M	84.2 (63.77, 104.56)	1.33 (1.08, 1.59)
Packaging containers (12)	cfu/cm^2	MB	<1 (–)**	0.50 (0.00, 1.19)
		Ent	<1 (–)	0.00 (–)
Olive fruit (36)	log_{10} cfu/g	MB	4.39 (4.08, 4.58)	1.33 (1.07, 1.60)
		Y/M	3.80 (3.57, 3.95)	1.00 (0.71, 1.29)
		LAB	3.75 (3.11, 3.99)	0.50 (0.23, 0.77)
		Ent	2.68 (2.18, 2.91)	1.50 (0.96, 2.03)
		CPS	<1.30 (–)	0.33 (0.16, 0.50)
		SRC	<1.30 (–)	1.00 (0.49, 1.51)
Olive brine (24)	log_{10} cfu/ml	MB	1.62 (<1.30, 1.84)	0.17 (0.00, 0.33)
		Y/M	<1.30 (–)	0.00 (–)
		LAB	<1.30 (–)	0.00 (–)
		Ent	<1.30 (–)	0.00 (–)
Handlers gloves (12)	cfu/cm^2	MB	2.04 (<1.30, 2.34)	1.40 (0.75, 2.05)
		Ent	<1 (–)	0.60 (0.00, 1.35)
Water (18)	cfu/100 ml	MB	14.30 (<10, 23.96)	0.83 (0.64, 1.02)
		Col	<10 (–)	0.00 (–)
		SRC	<10 (–)	0.00 (–)

**MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; Col, total coliforms.*

***CI 95% could not be estimated.*

distributions EKE can be considered for the derivation of distribution parameters (Clemen and Winkler, 1999). For the present study, the relative importance of each processing step was quantified by a group of 25 quality inspectors from the table olive sector together with scientists and public health authorities. A percentage from 0 to 100% was individually assigned to each processing step (% F_i) and a triangular distribution with three parameters; most probable number, minimum and maximum. These percentages (% F_i) were included in the model to weight the steps according to the experts' opinion. For the processing step F_i, the weighted PHSS values ($PHSS_w$) were calculated as:

$$PHSS_w = \left(\frac{D_{\max F_{TOT}}}{D_{TOT}}\right) + \left(\frac{\%F_i}{100}\right) x \left(\frac{PHSS_{F_i}}{100}\right) \quad (7)$$

It should be noted that $PHSS_{Fi}$ values provide a measure of the global variability in the elaboration process while $PHSS_w$ values are indicative of the individual contribution of each processing step to the overall quality and safety of the finished product.

Statistical Analyses

Boxplots including the main descriptive statistics (mean, standard deviation, 5th, 95th percentiles) were generated for each model output, i.e., $PHSS_{Fi}$ and $PHSS_w$ values. Descriptive statistics of the final distribution outputs were used to quantify model variability associated to the hygienic-sanitary conditions in each processing step. Uncertainty was considered using the 95% CI for the microbiological results and scores. Further, an ANOVA analysis was also performed to find significant differences between processing steps in relation to the PHSS values calculated ($p < 0.05$).

Further, Spearman correlation coefficients were obtained through a sensitivity analysis to identify the most relevant processing steps, samples and parameters that may exert an influence on the final product quality and safety. To avoid unrealistic results of the model, the Spearman's rank order correlation in the @Risk software was used to assume a previous high-dependence association between microbial loads found in olive fruits and brines ($r = 0.75$). The probabilistic model was run with a MonteCarlo simulation in @Risk v7.5 with 10,000 iterations.

RESULTS AND DISCUSSION

Hygienic-Sanitary Status of *Aloreña* Table Olive Processing

To evaluate the status of the hygienic-sanitary conditions throughout the *Aloreña* table olive processing, a total of 658 microbiological and physico-chemical data were obtained from brines, olive fruits, air environment, food-contact surfaces, food handlers, and water samples in three industries. Besides, finished packed table olives were characterized after processing just before commercialization. The mean concentrations together with the assigned scores to each processing step are represented in **Tables 1–5**.

Processing Step 1: Reception of Raw Materials and Fermentation

In **Table 1**, the status of olive brines and fruits once fermentation was completed indicated a relatively high concentration of MB and Y/M. Olive brines presented a mean value of 6.20 log_{10} cfu/ml of MB while Y/M concentration corresponded to 5.00

TABLE 5 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 5 (finished product).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Olive brine (48)	log_{10} cfu/ml	MB	3.08 (3.00, 3.14)	1.67 (1.52, 1.81)
		Y/M	3.20 (3.06, 3.31)	1.33 (1.10, 1.56)
		LAB	3.82 (3.48, 4.01)	1.33 (1.04, 1.62)
		Ent	<1.30 (–)**	0.17 (0.05, 0.28)
		CPS	<1.30 (–)	0.00 (–)
		SRC	<1.30 (–)	0.50 (0.15, 0.85)
Olive fruit (48)	log_{10} cfu/ml	MB	3.89 (3.65, 4.05)	0.83 (0.56, 1.11)
		Y/M	3.85 (3.70, 3.96)	1.33 (1.19, 1.48)
		LAB	4.35 (4.09, 4.52)	1.00 (0.75, 1.25)
		Ent	<1.30 (–)	0.17 (0.05, 0.28)
		CPS	<1.30 (–)	0.50 (0.35, 0.65)
		SRC	<1.30 (–)	0.50 (0.15, 0.85)
		LM	<−1.40 (–)	0.00 (–)
		Salm	<−1.40 (–)	0.00 (–)
Olive brine (24)	–	pH	4.23 (4.14, 4.32)	1.50 (1.11, 1.89)
	mEq/ml	FA	0.31 (0.28, 0.34)	0.50 (0.26, 0.74)
	% (w/v)	NaCl	5.44 (5.36, 5.52)	1.00 (0.75, 1.25)
	g/l	Sugar	2.70 (2.00, 3.40)	0.50 (0.35, 0.65)

**MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae, CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; LM, L. monocytogenes; Salm, Salmonella sp.; Col, total coliforms.*

***CI 95% could not be estimated.*

log_{10} cfu/ml. LAB concentration was also higher than 6 log_{10} cfu/ml. These microbiological values are in agreement with data reported by Arroyo-López (2007) for this type of table olive specialty in this step. In olive fruits, microbial loads were slightly lower though presence of SRC was detected at low levels (around 1 log_{10} cfu/g). Neither Ent nor CPS were detected in brines or fruits samples in this processing step. The absence of Ent was related with the low pH obtained after fermentation of fruits (Garrido-Fernandez et al., 1997). Air contamination was qualified as intermedium for MB (average count of 2.32 log_{10} cfu/m^3) while lower values were obtained for Y/M (1.75 log_{10} cfu/m^3). Regarding physico-chemical data, it should be noted that some deficiencies were denoted regarding pH values of brines, which were slightly higher than 4.3, meaning that these samples would not comply with the requirements stated in the international laws (Codex Alimentarius 1981, rev 2013; IOC, 2004), where maximum allowable pH is 4.3. On the contrary, data obtained for FA and salt can be considered as normal (Garrido-Fernandez et al., 1997; Arroyo-López, 2007). Finally, water samples presented unacceptable values of MB, Col and SRC though these two later groups were detected after samples enrichment.

Processing Step 2: Olive Washing and Cracking

After fermentation of fruits, olives were washed and cracked by industry. Cracking step is considered as a critical control point in the HACCP system since microbial hazards present in contaminated olive fruits can be spread during the cracking process to non-contaminated fruits, brines or food-contact surfaces. The microbiological quality of brines and fruits was very similar to the processing step 1 (**Table 2**). However, presence of Ent was observed at high levels in the hopper surfaces (mean = 1.86 log_{10} cfu/cm^2) and could have been probably transferred to olive brines since more than 4 log_{10} cfu/ml was observed in some of evaluated samples. These loads were also observed by other authors (Arroyo-López, 2007; Alves et al., 2012) ranging from 2.6 to 3.5 log_{10} cfu/ml in brines at the beginning of the fermentation period, but there is no information available on the surface of machinery, containers, operators, etc., in olive industry. The presence of Ent is not desired in table olives because they could jeopardize the stability and safety of finished products (Garrido-Fernandez et al., 1997). Air contamination was classified as "intermediate," according to the microbial concentrations obtained (100–300 cfu/m^3).

Processing Step 3: Selection and Addition of Olive Dressings

In this step, samples collected corresponded to air environment, conveyor belts, handlers' gloves, olive fruits, and olive dressings (red pepper, garlic and herbal mixture). Overall, high microbial counts of MB and Ent were obtained in samples from conveyor belts. Presence of Ent was detected in olive fruits (mean = 2.30 log_{10} cfu/g), and in olive dressings, being higher for the herbal mixture (mean = 6.45 log_{10} cfu/g). Addition of herbs and spices to olive fruits could imply an increase in the microbial load of finished products given the high concentrations of MB, Y/M, LAB, and Ent (Arroyo-López, 2007). Besides, product safety could be compromised since high concentrations of CPS were detected in herb samples (mean = 4.42 log_{10} cfu/g) together with the presence of SRC (**Table 3**). However, the influence

TABLE 6 | Scoring system assigned to the different physico-chemical and microbiological parameters analyzed and samples collected.

Type of sample	Parameters*	Units	Scores				Source**
Air environment	MB and Y/M	cfu/m^3	<10 (0)	10–100 (1)	101–300 (2)	>300 (3)	1
Food-contact surfaces	MB	cfu/cm^2	<1 (0)	1–10 (1)	11–100 (2)	>100 (3)	2
	Ent	cfu/cm^2	<1 (0)	1–5 (1)	5–10 (2)	>10 (3)	2
Olive fruits (semi-elaborated)	MB	cfu/g	$<10^3$ (0)	10^3–10^4 (1)	10^4-10^6 (2)	$>10^6$ (3)	3, 4, 5
	Ent	cfu/g	<20	21–50	51–100	>100	3, 4, 5
	LAB	cfu/g	$<10^3$ (0)	10^3–10^4 (1)	10^4–10^6 (2)	$>10^6$ (3)	3, 4, 5
	Y/M	cfu/g	$<10^3$ (0)	10^3–10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	CPS	cfu/g	<20 (0)	21–50	51–100	>100	3, 4, 5
	SRC	cfu/g	<20 (0)	–	–	≥20 (3)	3, 4, 5
Olive fruits (finished product) and olive dressings (garlic and red pepper)	MB	cfu/g	$<10^3$ (0)	10^3-10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	Ent	cfu/g	<20	21–50	51–100	>100	3, 4, 5
	LAB	cfu/g	$<10^2$ (0)	10^2–10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	Y/M	cfu/g	$<10^2$ (0)	10^2–10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	CPS	cfu/g	<20 (0)	21–50	51–100	>100	3, 4, 5
	SRC	cfu/g	<20 (0)	–	–	≥20 (3)	3, 4, 5
	LM	cfu/g	<1 /25g (0)	–	–	≥1 /25 g (3)	6
	Salm	cfu/g	<1 /25g (0)	–	–	≥1 /25 g (3)	6
Brines	MB	cfu/ml	$<10^2$ (0)	10^2-10^3 (1)	10^3-10^5 (2)	$>10^5$ (3)	3, 4, 5
	Ent	cfu/ml	<20	21–50	51–100	>100	3, 4, 5
	LAB	cfu/ml	$<10^2$ (0)	10^2–10^3 (1)	10^3–10^5 (2)	$>10^5$ (3)	3, 4, 5
	Y/M	cfu/ml	$<10^2$ (0)	10^2–10^3 (1)	10^3–10^5 (2)	$>10^5$ (3)	3, 4, 5
	pH	–	<4.0 (0)	4.0–4.2 (1)	4.2–4.3 (2)	>4.3 (3)	7
	FA	g/100 ml	>0.3 (0)	0.2–0.3 (1)	0.1–0.2 (2)	<0.1 (3)	7
	NaCl	% (w/v)	>6.0 (0)	5.5–6.0 (1)	5.0–5.5 (2)	<5.5 (3)	7
	Sugar	% (g/l)	<2.0 (0)	2.0–9.0 (1)	9.0–19.0 (2)	>19.0 (3)	7
Olive dressing (herbs)	SRC	cfu/g	<20	21–100	101–10^3	$>10^3$	3, 4, 5
Water	MB	cfu/100 ml	<1 (0)	1–50 (1)	51–100 (2)	>100 (3)	8
	Col	cfu/100 ml	<1 (0)	–	–	≥1 (3)	8
	SRC	cfu/100 ml	<1 (0)	–	–	≥1 (3)	8

**MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; FA, free acidity; Col, total coliforms; LM, L. monocytogenes; Salm, Salmonella sp.*

***1 (Al Dagal et al., 1992); 2 (Sneed et al., 2004); 3 [Codex Standard for Table Olives (Codex 66-1981, 2013, Codex Alimentarius Commission, 1981)]; 4 (Trade Standard Applying to Table Olives, IOC, 2004); 5 [Code des Bonnes Pratiques Loyales pour les Olives de Table (Federation des Industries Condimentaires de France, 2000)]; 6 [Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, Commission Regulation, 2007); 7 (Royal Decree 1230/2001, of 8 November, approving the Technical-sanitary Regulation for the elaboration, distribution and sale of table olives); 8 [Royal Decree 140/2003 (Royal Decree, 2003) of 7 February, establishing the sanitary quality criteria of water for human consumption].*

of seasoning material in table olive processing has not been studied in detail in spite of their considerable influence on quality and safety of finished products. Samples from food handlers presented relatively low counts of MB. Ent were not detected in handlers' gloves.

Processing Step 4: Packaging Process

Table 4 represents the microbial counts obtained in the packaging step. A substantial reduction in mean counts were observed in comparison to the previous steps. This could be attributed to the inhibitory effect of salt concentration and pH together with the renovation of brines which imply a reduction in the microbial load of olive fruits. However, low counts of Ent were observed in fruits (mean = 2.68 $\log_{10}$ cfu/g) that could probably be associated to the high concentrations detected in olive dressings and transferred to this step. Olive brines had good microbiological quality as well as water samples. CPS and SRC were not detected in any sample.

Processing Step 5: Finished Product

Finished products just before commercialization presented lower microbial concentration of all groups analyzed which means that contamination during processing can be sporadic and product formulation (especially salt, pH values, and addition of preservatives) does not allow microbial growth during shelf life. In **Table 5**, it can be observed that concentrations were below 4 $\log_{10}$ cfu/g in all samples evaluated. Further, Ent, SRC and CPS were not detected in any sample. Absence or low levels of Ent in finished product is in line with data obtained by other authors which reflect their survival in olive packaging only during the first days (Bautista-Gallego et al., 2010; Romero-Gil et al., 2016). LM and Salm were not detected in any sample of fruits and brines in the finished product. This data is in concordance with the study carried out by Medina et al. (2016), who related the inhibition of diverse food-borne pathogens (among them *Listeria* and *Salmonella*) in *Aloreña de Málaga* brines by the presence of diverse phenolic compounds.

It should be noted that for some samples, pH values and salt concentrations exceeded the recommended limits for table olives (pH > 4.3; NaCl < 6%) which could imply that halotolerant or acidic-resistant microorganisms could proliferate during storage if they are previously present in the intermediate fruits and /or brines. Further, mean content of residual sugar in olive fruits was 2.70 g/l, which could support microbial growth. This is particularly relevant for olive production, since yeasts have the capacity to produce refermentation in presence of residual sugars (Loureiro and Malfeito-Ferreira, 2003). In this context, it is highly important to reduce yeasts concentration during the table olive processing in order to improve product stability and shelf-life (Alves et al., 2012). Bautista-Gallego et al. (2013) related yeasts as the main microbial agent causing instability of *Aloreña de Málaga* packaging at salt concentration above 5.0%, while Romero-Gil et al. (2016) point to LAB as spoilage microorganisms when the salt concentration was below this critical level.

Probabilistic Assessment of Hygiene and Safety of *Aloreña de Málaga* Table Olives

Simulation Results of PHSS and $PHSS_w$ Values

In the present study, a decision-making scoring system was suggested to operationalize hygienic-sanitary conditions in the *Aloreña de Málaga* table olives processing. The degree of fulfillment and the variability in the hygienic and safety conditions was quantified at each processing step ($PHSS_{Fi}$) as well as for the global process through the calculation of $PHHS_{FTOT}$. Besides, the relative importance of processing conditions was quantified by experts' elicitation. This information served to estimate the $PHSS_w$ values.

Figure 2 shows the simulation results of $PHSS_{Fi}$ and $PHSS_{FTOT}$ outputs. The mean value of the global Performance Hygiene and Safety Score for the *Aloreña de Málaga* table olives processing ($PHHS_{FTOT}$) was 64.82% (90th CI: 52.78–76.39%) indicating a variation in the hygienic practices in the evaluated processing steps among different industries. Washing and cracking, and selection and addition of olive dressings were detected as the most deficient steps since the lowest $PHSS_{Fi}$ values were obtained ($p < 0.05$) (mean = 53.02 and 56.62% respectively). Especially for washing and cracking, variability in processing conditions among facilities was the highest (90th CI: 26.67–80.00%) and high contamination of brines and fruits were obtained. Packaging and finished products showed higher $PHSS_{Fi}$ values (mean > 73%) probably attributed to product formulation (combination of low pH and high NaCl levels) together with the addition of new brines and preservatives that contributed to a reduction of microbial contamination at the packaging step.

$PHHS_w$ values were mainly based on the elicitation scores assigned by different experts from the *Aloreña de Málaga* table olive processing sector. Further, triangular distributions with minimum, most probable and maximum scores for each processing step were adjusted. Distributions were used as modeling inputs to estimate the individual contribution of the processing steps to the overall hygienic-sanitary conditions of finished products. In **Table 7**, descriptive statistics and percentiles

TABLE 7 | Elicitation scores (%) assigned by individual experts ($n = 25$) from the *Aloreña de Málaga* table olive sector.

Processing step	Distribution	Mean	S.D.	5th Perc	95th Perc
Reception of raw material and fermentation	RiskTriang(5;10;40)	16.49	6.18	8.02	27.64
Olive washing and cracking	RiskTriang(5;20;30)	17.10	4.61	9.30	24.49
Selection and addition of olive dressings	RiskTriang(10;20;40)	21.46	5.29	13.49	30.84
Packaging process	RiskTriang(10;20;30)	18.86	3.92	12.66	25.61
Finished product	RiskTriang(5;40;40)	26.12	6.74	13.79	36.22

Parameters used for triangular distributions are shown together with simulated statistics.

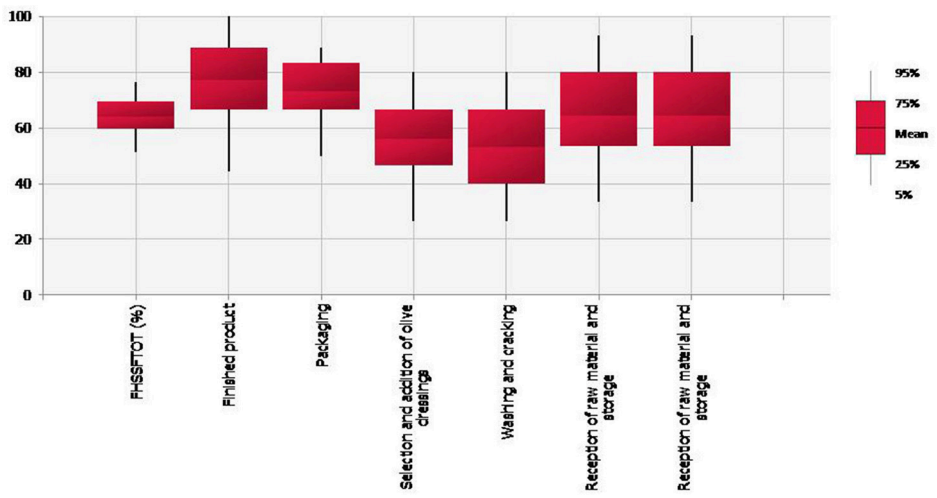

FIGURE 2 | Boxplot representing the mean, 5, 25, 75, and 95th values of the Performance Hygiene and Safety Score at the different processing steps ($PHSS_{Fi}$) together with the global $PHSS_{FTOT}$ (%).

of simulated distributions indicated that the finished product was assigned by experts as the most relevant step from a hygiene and safety point of view, having the highest 95th percentile (36.22%) followed by the selection and addition of olive dressings (30.84%). In contrast, reception of raw materials and fermentation, olive washing and cracking and packaging steps had the lowest 95th percentiles (24.49–27.64%). The main premise behind an expert elicitation method is that the method employed incorporates the knowledge and experience of the experts, and reduces the judgment biases. In the present study, the use of questionnaires allowed to collect information from quality inspectors of the table olive sector. Expert opinions can be used to address important questions and uncertainties in risk analysis. However, one of the limitations of expert elicitation is that sometimes experts may not describe accurately their actual knowledge so that data selection should be taken with caution.

In **Figure 3**, the relative contribution of each processing step on the $PHSS_w$ was represented, according to the values provided by the experts (**Table 7**). Significant differences in $PHSS_w$ values were obtained between packaging process and finished products, and the remaining processing steps ($p < 0.05$). The mean value of $PHSS_w$ was 65.53% (90th CI: 53.12–77.52%), very similar with $PHSS_{FTOT}$ with 64.82%. As above mentioned for $FHSS_{FTOT}$, the final processing steps obtained higher values for $PHSS_w$ being the finished product the most relevant one (mean = 18.44%; 90% CI: 10.34–25.33%). However, it should be noted that $PHSS_w$ values are influenced by the weighting percentage assigned by the experts. In this case, the final steps were considered highly relevant for preserving the stability of the finished product and its shelf-life extension.

Sensitivity Analysis of PHSS and $PHSS_W$ Values on the Type of Sample and Processing Step

In **Figure 4**, Spearman correlation coefficients describing the relative influence of the type of sample on the $PHSS_{FTOT}$ and on the $PHSS_w$ are represented. As $PHSS_{FTOT}$ were calculated without weighting the processing steps (all of them were considered equally relevant for final product quality and safety), correlation coefficients were higher for the primary steps which corresponded to the most contaminated samples. Particularly, the microbiological quality and safety of used brines presented a high correlation (−0.30 for brines used during the reception of raw materials and storage; and −0.28 for brines used during washing and cracking of table olives) with the final $PHSS_{FTOT}$ values, followed by results obtained in processing step 3 (selection and addition of olive dressings) for intermediate fruits and olive dressings.

On the contrary, for $PHSS_w$ values, (**Figure 4B**) the finished product presented the highest correlations (fruits, brines and physico-chemical values) since this step contributed mostly to the increase of $PHSS_w$.

Sensitivity analysis was also performed on the relative variation of each type of sample on the mean $PHSS_{FTOT}$ and $PHSS_w$ values (**Figure 5**). It can be concluded that intervention measures focused on reducing the contamination of washing brines (processing step 2) could lead to an improvement of $PHSS_{FTOT}$ value to 67.03 %. On the contrary, contamination of fruits during washing and cracking could also lead to a reduction of $PHSS_{FTOT}$ values to 60.58%.

Regarding $PHSS_w$, in **Figure 5B**, physico-chemical values and contamination of brines and fruits in the processing step 5 (finished product) produced the widest variation of $PHSS_w$ values. However, as seen in **Table 5**, contamination of brines and fruits were relatively lower than in previous steps, being influenced by the addition of olive dressings as well as by product formulation. It should be remarked that corrective measures implemented during washing and cracking can be equally effective on the $PHSS_w$ (68.11%). It was also identified that cleaning of washing hoppers at processing step 2 could increase the final $PHSS_w$ value up to 68.16%.

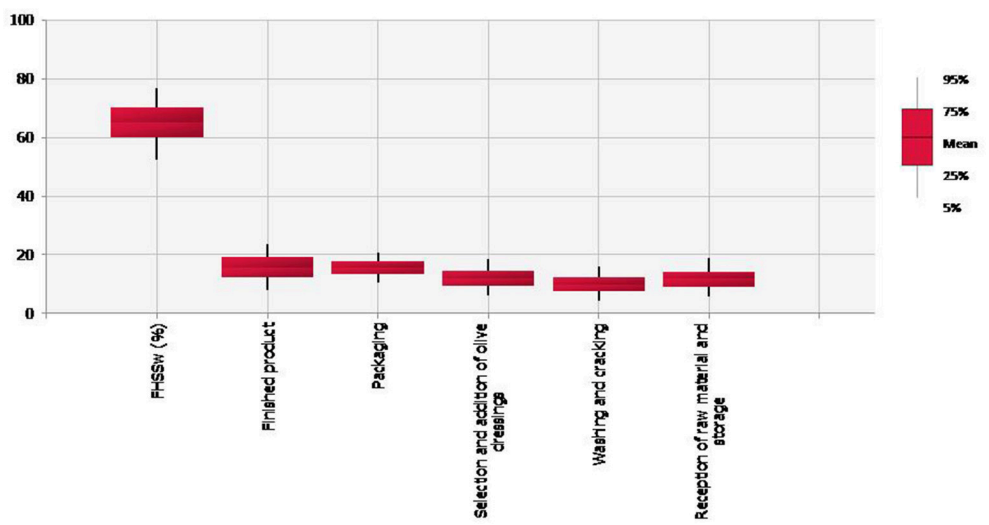

FIGURE 3 | Boxplot representing the mean, 5, 25, 75, and 95th values of the individual contribution of the processing steps on the weighted Performance Hygiene and Safety Score $PHSS_W$ (%).

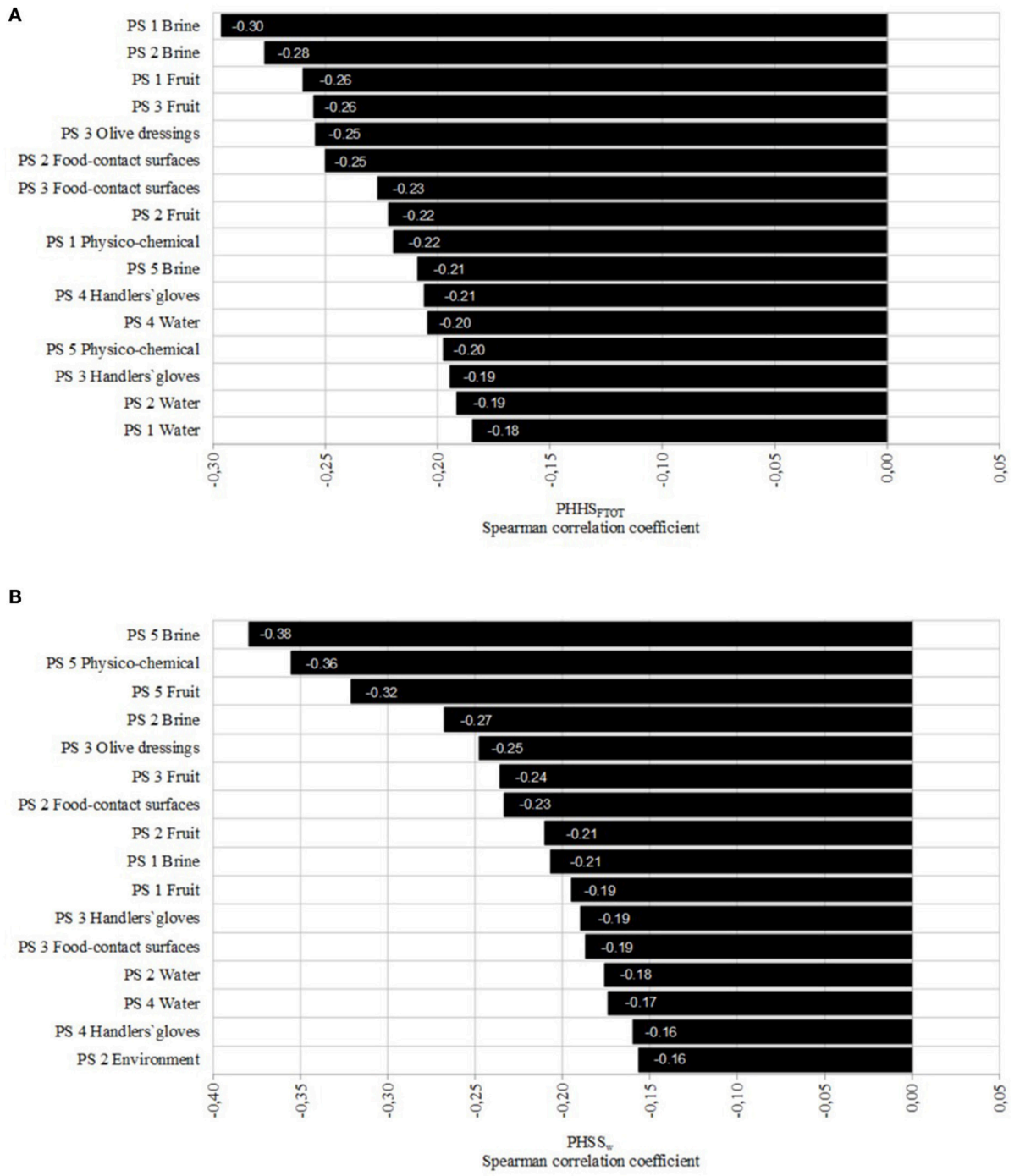

FIGURE 4 | Spearman correlation coefficients describing the relative influence of the type of sample on the final global Performance Hygiene and Safety Score ($PHSS_{FTOT}$) **(A)** and the weighted Performance Hygiene and Safety Score ($PHSS_w$) **(B)**. PS stands for the processing step.

In **Figure 6**, a direct correlation was found between simulated $PHSS_w$ values and relative contributions of each processing step. Simulated results showed that the proportion of directly correlated values was higher for steps 2 (66.4%) and 5 (70.1%). Packaging was identified as the step with lesser proportion of directly correlated values with $PHSS_w$ (57.4%).

To date, there are not probabilistic tools based on the application of FQSMS in the table olive sector. There are other tools in literature in which a systematic analysis of microbial counts was used to assess the degree of performance of a FQSMS (Jacxsens et al., 2009; Lahou et al., 2014). These approaches are based on a selection of critical sampling location, selection

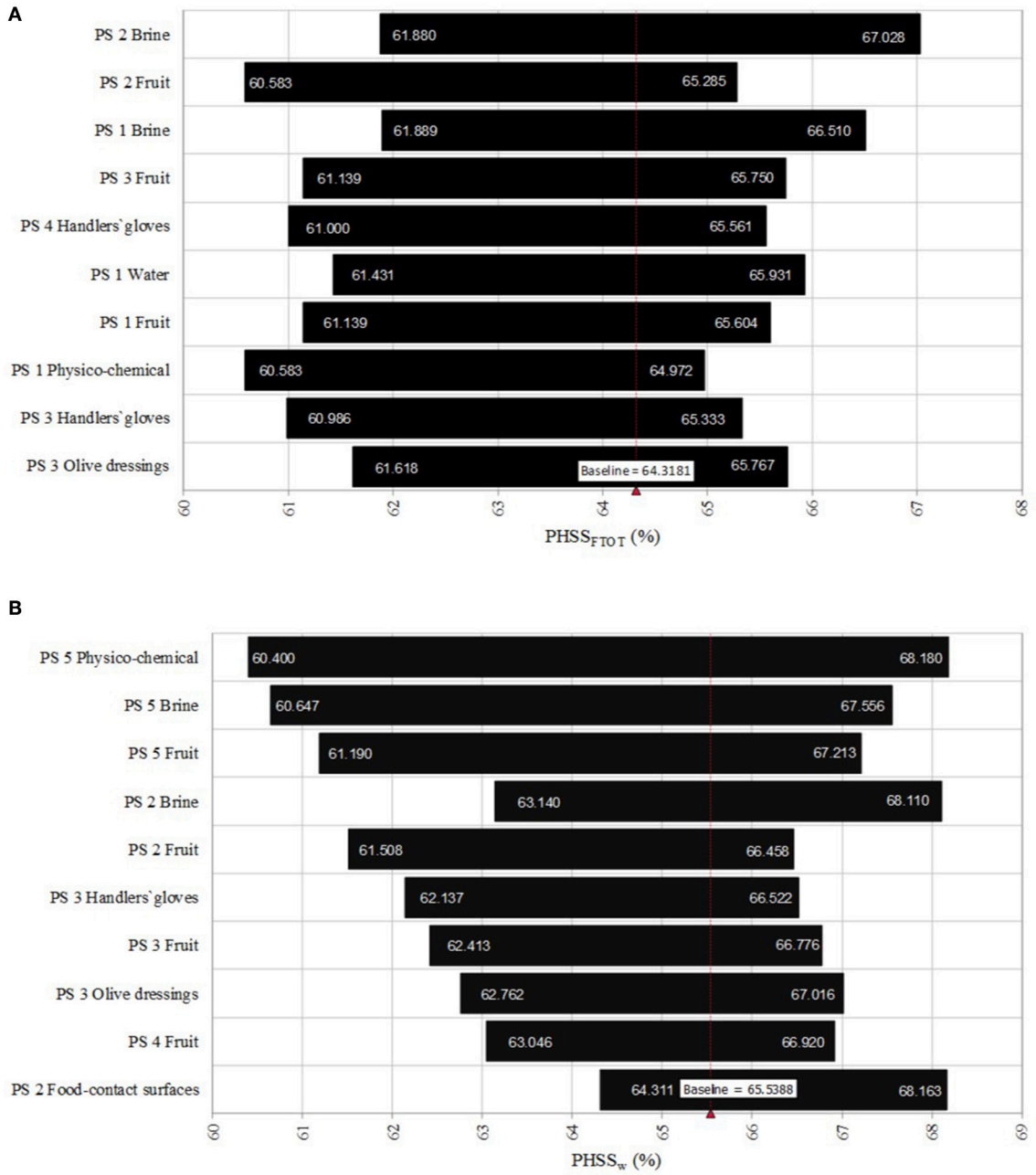

FIGURE 5 | Results of the sensitivity analysis describing the relative influence of the type of sample on the variation of the mean value for the global Performance Hygiene and Safety Score ($PHSS_{FTOT}$) **(A)** and the weighted Performance Hygiene and Safety Score ($PHSS_W$) **(B)**. PS stands for the processing step.

of microbial parameters, assessment of sampling frequency, selection of sampling and analytical methods and data processing and interpretation. Different microbial safety levels are assigned according to the compliance with legal criteria for both microbial hygiene and safety. The approach followed in the present study is in agreement with the principle behind Microbial Assessment Schemes (MAS) in which low concentration of microorganisms and small variability indicate an effective FQSMS (Sampers et al., 2010; Luning et al., 2011).

In conclusion, it is suggested that corrective measures should be focused on reducing the microbial contamination of brines and fruits at primary steps, together with the implementation of novel treatments on olive dressings (irradiation, scalding, ozonization, etc.) to reduce their microbial load since contamination can persist in brines and fruits during table olive processing. According to the suggested approach, these preventive measures can be equally or even more effective than modifying product formulation to

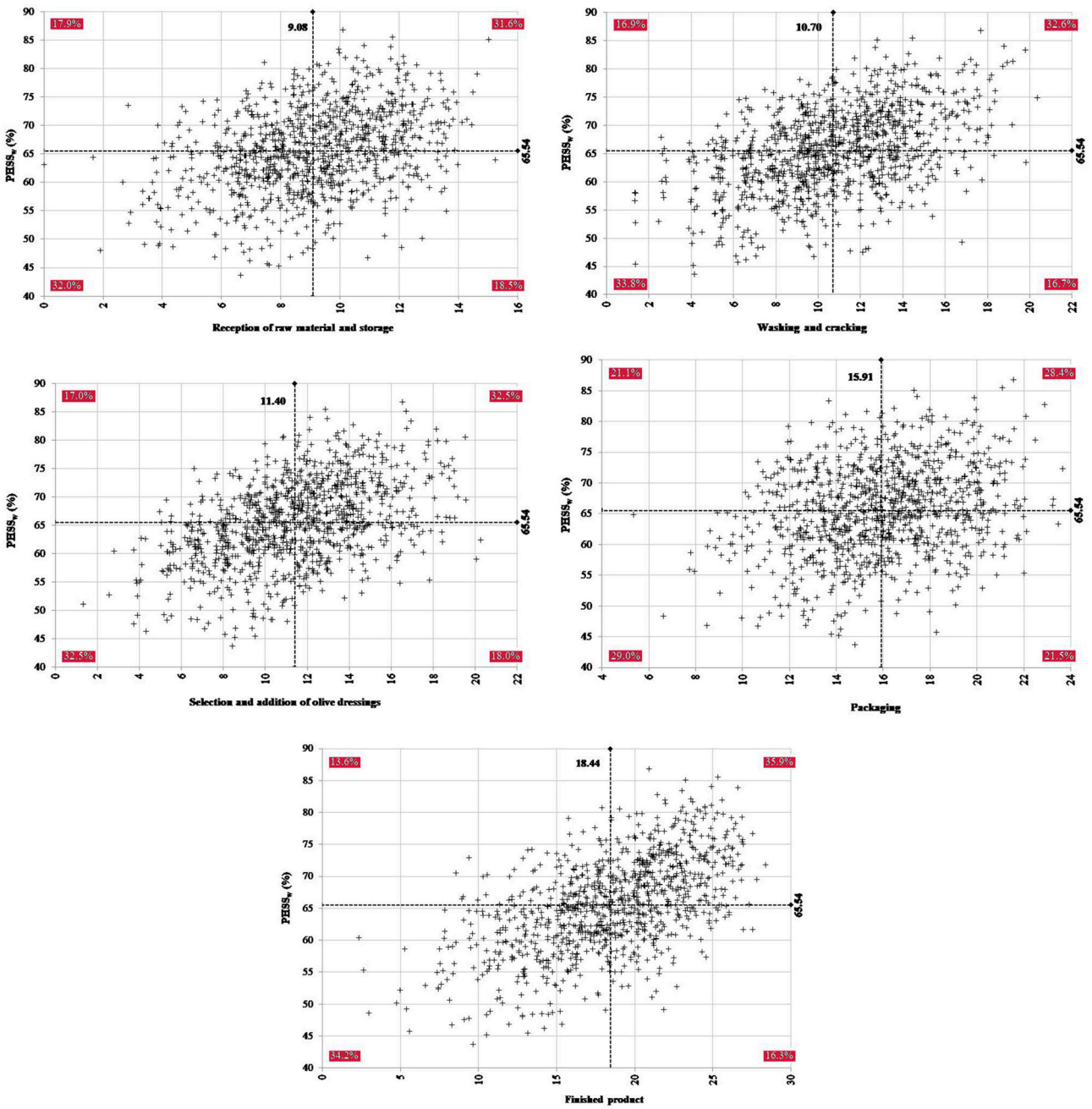

FIGURE 6 | Relationship between simulated PHSS$_W$ values and individual contributions of the evaluated processing steps.

lower pH values and higher salt concentrations. In addition, industry could reduce the levels of salt and preservatives in packaging producing a healthier product. The results presented are currently integrated within a software tool which will provide stakeholders with an easy-to-use, flexible and useful probabilistic decision-making scoring system for the *Aloreña de Málaga* table olive food sector. Furthermore, the approach can be extended to other olive varieties and elaboration methods including alternative treatments and steps as long as the information about scores weighing becomes available.

AUTHOR CONTRIBUTIONS

MR, FR-G, EM, VR, AP, and GP-I executed the experimental work and microbiological and physico-chemical analysis. FA-L, FR, RG-G, and AV planning the experiment and written the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ANEXO I. PROTOCOLO PARA LA TOMA DE DECISIONES RELACIONADAS CON LA GESTIÓN DE LA CALIDAD Y SEGURIDAD ALIMENTARIA EN LAS EMPRESAS ELABORADORAS DE ACEITUNA DE MESA. PI 3731/2016.

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Actualmente las empresas del sector de la aceituna de mesa realizan todos los procesos de gestión de la calidad, seguridad alimentaria y análisis de puntos críticos (APPCC) de una manera empírica, subjetiva, difícilmente evaluable y sin el suficiente rigor científico.

El protocolo para la toma de decisiones de gestión de calidad y seguridad alimentaria en empresas elaboradoras de aceituna de mesa, en adelante *el protocolo*, que se describe en el presente documento se relaciona con metodologías y técnica basadas en procedimientos científicos para evaluar sus procesos de una manera cuantitativa, determinando que etapas son susceptibles de ser mejoradas. La adopción de este tipo de enfoque está en línea con las recomendaciones planteadas por organizaciones internacionales como EFSA, FAO/OMS o el *Codex Alimentarius* sobre los cambios en las estrategias de control basadas en la aparición de peligros en el producto final hacia el establecimiento de medidas de gestión del riesgo.

El protocolo tiene su núcleo central en un sistema de ponderaciones y análisis matemáticos para cada una de las etapas que comprenden la elaboración del producto y las condiciones de procesado en la industria (recogida de los frutos, lavado, fermentación, conservación, envasado, etc.), a partir de analíticas de tipo: a) microbiológico (recuentos de microorganismos alterantes y detección de patógenos en muestras de superficies, salmueras, agua, ambiente y fruto); y b) físico-químico en muestras de salmuera y productos (valores de pH, sal, acidez libre y combinada, azúcares reductores, etc.).

Se conocen trabajos en el estado de la técnica relacionados con la implementación de modelos matemáticos integrales para calcular el riesgo microbiológico (ver por ejemplo: Risk Ranger (Sumner y col., 2005: http://www.sciencedirect.com/science/article/pii/S0168160505003284) dirigido a carne de vacuno, ovino y caprino; o sQMRA (Evers y Chardon, 2010: http://www.sciencedirect.com/science/article/pii/S0956713509001893) utilizado con éxito en muestras de filete de pollo, filete americano (carne picada cruda con mayonesa) y huevos de mesa), sin embargo, se trata de propuestas de un alcance más limitado que el del protocolo que se aquí se describe y que además no se han diseñado específicamente para empresas elaboradoras de aceitunas de mesa.

Más concretamente, y aunque ya se han divulgado parcialmente diversos aspectos del protocolo que a continuación se definirá (ver por ejemplo: Ruiz Bellido, MA. 2014. Análisis y diseño de sistemas de cuantificación de las condiciones higiénico sanitarias del proceso de elaboración de la aceituna Aloreña de Málaga. Trabajo Fin de Máster en AgroAlimentación. Julio 2014. Universidad de Córdoba; Ruiz Bellido MA. *et al.* 2016. P3-129. *Development of Decision–Support Systems Based on Physico-chemical and Microbiological Data for Improvement of the Quality and Safety of Aloreña de Málaga Table Olives*. IAFP. Sant Louis. Missouri. 30 Julio a 3 de Agosto 2016; o Díaz Ruiz L. Desarrollo de sistemas de toma decisiones de basados en la caracterización físico-química y microbiológica de procesos y productos de elaboración de la aceituna de mesa "*Aloreña de Málaga*". Trabajo Fin de Grado. Julio 2015. Universidad de Córdoba), esta es la primera vez que se recoge en un único documento una descripción tan detallada de dicho protocolo, que incluye una lista completa de etapas y parámetros a analizar, así como la inclusión de ponderaciones en base a opiniones de expertos y la automatización, a través de una hoja de cálculo, del modelo matemático gracias al cual se obtienen los resultados.

Las principales ventajas de este novedoso protocolo tienen que ver con la rapidez y eficacia en la toma de decisiones, de tal forma que:

- Permite introducir o eliminar fases del proceso en caso necesario o actualizar las valoraciones en base a los resultados que se vayan generando a partir de cada analítica,

- Permite cuantificar el efecto relativo de cada una de las fases que forman parte de su proceso sobre la calidad higiénica del producto final y establecer medidas correctoras en su caso.

DESCRIPCIÓN DEL PROTOCOLO PARA LA TOMA DE DECISIONES

El protocolo comprende cuatro etapas (a, b, c y d):

Etapa a): consistente en tomar muestras procedentes del ambiente de procesado, superficies de contacto con alimentos, manipuladores, frutos no envasados, salmueras, agua, especias y producto final en las distintas fases que tienen lugar en la producción de aceitunas de mesa, singularmente:

Fase 1: Recepción y almacenamiento de la materia prima

Fase 2: Lavado y partido de los frutos

Fase 3: Inspección y adición de aliños

Fase 4: Envasado

Fase 5: Expedición del producto final

Etapa b): consistente en realizar una batería de análisis (variables de entrada o *inputs*) a las muestras tomadas en las distintas fases de la etapa a), singularmente:

1. *Microbiológicos*: aerobios mesófilos, mohos y levaduras, bacterias ácido-lácticas, Enterobacterias, Clostridios sulfito reductores, *Staphylococcus* coagulasa positivos, coliformes, *Listeria monocytogenes* y *Salmonella* spp.

2. *Físico-químicos*: pH, acidez libre y combinada (mEq/ml), contenido en azúcares (g/L), y contenido en sal (%).

En un ejemplo de realización, el protocolo se utiliza para el control de empresas productoras de aceituna *Aloreña de Málaga*, y los análisis se aplican sobre 255 muestras recogidas en las distintas fases de producción. Sin embargo, también es posible su adaptación y aplicación sobre otras variedades de aceituna, y la incorporación de análisis

de parámetros adicionales como presencia de pesticidas, alérgenos, porcentaje de alambrado, etc.

Etapa c): consistente en obtener las variables de respuesta (*outputs*) que se corresponden con valores numéricos que representan el grado de cumplimiento relativo a la calidad higiénica e inocuidad del proceso y producto final.

Para ello, los resultados cuantitativos obtenidos a partir de cada tipo de análisis son asociados con una serie de "puntuaciones" o "*scores*" que oscilan desde un valor 0 (que indica la mejor calidad higiénica, es decir el mayor grado de cumplimiento) hasta un valor de 3 (puntuación máxima, que indica la peor calidad higiénica/sanitaria posible). Este sistema de puntuaciones está basado en:

i) la legislación nacional e internacional específica de la aceituna de mesa (ver Tabla 1);

ii) legislación referente a condiciones higiénico-sanitarias en las industrias de alimentación (ver Tabla 1); y

iii) el propio conocimiento y experiencia que los investigadores y expertos tienen del sector para aquellos parámetros en los que no hay documentación o legislación al respecto pero sería aconsejable de incluir.

Tabla 1. Normativa y publicaciones científicas en las que se basa el sistema de ponderaciones

Tipo de Muestra	**Referencia o legislación aplicable para la determinación de las ponderaciones**
Fisico-química de salmueras	· REAL DECRETO 1230/2001, de 8 de noviembre, por el que se aprueba la Reglamentación técnico-sanitaria para la elaboración, circulación y venta de las aceitunas de mesa.
Frutos no destinados a consumo humano	· CODEX 66-1981 rev 2013 · TRADE STANDARD APPLYING TO TABLE OLIVES (COI, 2004) · GUÍA DE GESTIÓN DE LA CALIDAD DE LA INDUSTRIA DE ACEITUNAS DE MESA (COI, 2005) · CODE DES BONNES PRATIQUES LOYALES POUR LES OLIVES DE TABLE (FEDERATION DES INDUSTRIES CONDIMENTAIRES DE FRANCE, 2000)
Frutos destinados a consumo humano	· CODEX 66-1981 rev 2013 · TRADE STANDARD APPLYING TO TABLE OLIVES (COI, 2004) · GUÍA DE GESTIÓN DE LA CALIDAD DE LA INDUSTRIA DE ACEITUNAS DE MESA (COI, 2005) · CODE DES BONNES PRATIQUES LOYALES POUR LES OLIVES DE TABLE (FEDERATION DES INDUSTRIES CONDIMENTAIRES DE FRANCE, 2000)

Ambiente	AL DAGAL M, MO O, FUNG DYC AND KASTNER C. A CASE STUDY OF THE INFLUENCE OF MICROBIAL QUALITY OF AIR ON PRODUCT SHELF LIFE IN A MEAT PROCESSING PLANT. DAIRY FOOD ENVIRON SANITAT 12:69–70 (1992).
Patógenos	REGLAMENTO (CE) NO 2073/2005 DE LA COMISIÓN DE 15 DE NOVIEMBRE DE 2005 RELATIVO A LOS CRITERIOS MICROBIOLÓGICOS APLICABLES A LOS PRODUCTOS ALIMENTICIOS
Superficies	DECISIÓN DE LA COMISIÓN DE 6 DE NOVIEMBRE DE 2006 POR LA QUE SE DEROGAN DETERMINADOS ACTOS DE APLICACIÓN RELATIVOS A LA HIGIENE DE LOS PRODUCTOS ALIMENTICIOS Y A LAS NORMAS SANITARIAS QUE REGULAN LA PRODUCCIÓN Y COMERCIALIZACIÓN DE DETERMINADOS PRODUCTOS DE ORIGEN ANIMAL DESTINADOS AL CONSUMO HUMANO
Aguas destinadas a consumo humano	REAL DECRETO 140/2003, DE 7 DE FEBRERO, POR EL QUE SE ESTABLECEN LOS CRITERIOS SANITARIOS DE LA CALIDAD DEL AGUA DE CONSUMO HUMANO.

Etapa d): consistente en aplicar un modelo matemático a partir de las puntuaciones para cada tipo de muestra y fase correspondiente, e incluye las siguientes operaciones:

Operación 1. Cálculo del porcentaje del grado de cumplimiento de higiene y seguridad para cada fase aplicando la Ecuación 1:

$$\%cump = 1 - \left(\frac{p_i}{p_{maxi}} \right) x100 \qquad \text{Ecuación 1}$$

siendo *pi* la puntuación empírica obtenida en la fase *i* de las analíticas realizadas; y p_{maxi} la máxima puntuación teórica alcanzable en la fase *i* (peor calidad higiénica para los diferentes parámetros evaluados) en función de todos los parámetros físico-químico o microbiológicos que se analizan en la misma.

Operación 2. Cálculo del porcentaje de cumplimiento higiénico y de seguridad del conjunto de todas las fases, es decir el cumplimiento BASE global para las muestras procedentes de la industria, proporcionando información relativa a la variabilidad existente en las condiciones de procesado (*inter* e *intra*-empresa), aplicando la Ecuación 2:

$$\%cump = 1 - \left(\frac{p_{tot}}{p_{maxtot}} \right) x100 \qquad \text{Ecuación 2}$$

Donde:

$p_{tot} = p1 + p2 + p3 + ... + p8;$ siendo *p1* la puntuación alcanzada en la fase 1; *p2* la obtenida en la fase 2, etc.

$p_{maxtot} = p_{max}\ 1 + p_{max}\ 2 + p_{max}\ 3 + ... + p_{max}\ 8;$ siendo p_{max} 1 la puntuación máxima posible en la fase 1; p_{max} 2 la máxima posible en la fase 2 etc y p_{maxtot} la puntuación máxima total posible.

Operación 3. Establecimiento de una ponderación a cada una de las fases del proceso mediante el cálculo del porcentaje de cumplimiento PONDERADO global relacionada con la contribución individualizada de cada fase del proceso a la higiene y seguridad del producto final. Dicha variable se calcula mediante la Ecuación 3:

$$cumppond(\%) = \left(\frac{p_{max}}{p_{tot}}\right) + \left(\frac{p_{fas}}{100}\right) x \left(\frac{cump}{100}\right)$$

Ecuación 3

siendo p_{max} es la puntuación máxima posible de cada una de las fases del proceso; p_{tot} la puntuación alcanzada en cada una de las fases del proceso; y p_{fas} el porcentaje de importancia relativa de cada fase otorgado mediante opinión de expertos

Para poder establecer las ponderaciones correspondientes, el modelo permite introducir una serie de porcentajes que son asignados por expertos pertenecientes al sector y los propios investigadores, en función del peso que se considera que cada fase tiene sobre el proceso global en función de la opinión de expertos. Estos porcentajes consideran la variabilidad entre los distintos expertos mediante una distribución de tipo triangular, que tiene como parámetros el valor mínimo, más probable y máximo de cada fase del proceso.

El modelo matemático del protocolo que aquí se describe puede ser implementado en una hoja de cálculo MS Excel, que a su vez permite la incorporación de un software de análisis de riesgo, por ejemplo el software @Risk v7.5 (Palisade Corporation), que utiliza una simulación de MonteCarlo (#10000 iteraciones). A través de la simulación de dicho software, y de forma automatizada, a partir de los resultados de las Ecuaciones 2 y 3, se puede, entre otras opciones:

-obtener representaciones del porcentaje de cumplimiento BASE, que permite la determinación y cuantificación de la variabilidad del proceso productivo en cada una de las

fases, así como la variabilidad global. Esta información es de vital importancia para el empresario ya que es un indicador de la reproducibilidad de las condiciones de elaboración; e

-obtener representaciones del cumplimiento PONDERADO que representa la importancia relativa de cada fase a la higiene del proceso final, lo que permite adaptar estas variables y cuantificar el efecto de posibles medidas correctoras.

Sin embargo, también es posible su incorporación a un modelo matemático integral tal y como los que se conocen en el estado de la técnica o a una aplicación web que pueda ser utilizada y gestionada fácilmente por los potenciales usuarios. De esta forma, la propia industria podría volcar fácilmente los resultados de sus analíticas microbiológicas y físico-químicas, devolviendo el grado de cumplimiento que la empresa tiene en cada una de las etapas de desarrollo de sus productos y en el global. La aplicación podría trabajar asimismo como un asesor científico, ya que se podría introducir un sistema de alarmas en el que se notifica a la empresa cuando se corre un riesgo sanitario, se incrementa la probabilidad de sufrir una alteración, o se tienen errores recurrentes. Está herramienta matemática se podría implementar también dentro de los sistemas de APPCC y normas de calidad que tienen las empresas y en sus herramientas de gestión y software de ERP (Enterprise Resource Planning) como un módulo dentro de los mismos.

Ejemplo de realización

Ejemplo 1. Aplicación del protocolo para la toma de decisiones relacionadas con la gestión de la calidad y seguridad alimentaria en empresas elaboradoras de aceitunas de mesa de la variedad *Aloreña de Málaga*.

Visitas realizadas durante el período 2014-2016 a tres empresas representativas del sector productivo de aceitunas *Aloreña de Málaga* permitieron la toma de muestras procedentes de las distintas fases del proceso: ambiente de procesado, superficies de contacto con alimentos, manipuladores, frutos no envasados, salmueras, agua, especias y producto final. En total se obtuvieron 255 muestras sobre las que se realizaron un total de 1080 analíticas. Los resultados permitieron establecer las puntuaciones a tener en cuenta

para aplicar el modelo matemático que se describe en la etapa d) de la descripción del protocolo.

La Tabla 2 recoge las puntuaciones asignadas para los distintos niveles de cumplimiento para los parámetros elegidos para determinar el nivel de higiene de las distintas fases productivas en las muestras obtenidas. Dichas puntuaciones fueron establecidas por expertos en función de los valores obtenidos.

Tabla 2. Puntuaciones fijadas para los distintos parámetros analizados en la toma de muestras de: control de superficies, control ambiental, fruto (cadena de elaboración y producto final), salmuera, aliños y parámetros físico-químicos. Las puntuaciones quedan asignadas en cada caso entre paréntesis a los valores cuantitativos obtenidos de las analíticas.

Superficies	***Rango concentración (ufc/cm^2)***			
AMT	Ausencia/cm^2 (0)	1-10 (1)	11-100 (2)	>100 (3)
Enterobacterias	Ausencia/cm^2 (0)	1 (1)	2 (2)	>2 (3)
Ambiente	***Rango concentración (ufc/m^3)***			
AMT	Ausencia/m^3 (0)	1-100 (1)	101-300 (2)	>300 (3)
Mohos y levaduras	Ausencia/m^3 (0)	1-100 (1)	101-300 (2)	>300 (3)
Fruto (cadena elaboración)	***Rango concentración (ufc/g)***			
(aun no destinados a consumo humano)				
AMT	<10^3 (0)	10^3-10^4 (1)	10^4-10^6 (2)	>10^6 (3)
Enterobacterias	<20 (0)	20-50 (1)	51-100 (2)	>10^2 (3)
BAL	<10^3 (0)	10^3-10^4 (1)	10^4-10^6 (2)	>10^6 (3)
Mohos y levaduras	<10^3 (0)	10^3-10^4 (1)	10^4-10^5 (2)	>10^5 (3)
Staphylococcus coagulasa +	Ausencia/25g (0)	1-50 (1)	51-100 (2)	>100 (3)
Clostridium	Ausencia/25g (0)	Presencia/25g (3)		
Fruto (Envasado)	***Rango concentración (ufc/g)***			
(destinados a consumo humano)				
AMT	<10^3 (0)	10^3-10^4 (1)	10^4-10^5 (2)	>10^5 (3)
Enterobacterias	<20	20-50 (1)	51-100 (2)	>10^2 (3)
BAL	<100 (0)	10^2-10^4 (1)	10^4-10^5 (2)	>10^5 (3)
Mohos y levaduras	<100 (0)	10^2-10^4 (1)	10^4-10^5 (2)	>10^5 (3)

Esporulados	Ausencia/25g (0)	10-50 (1)	51-100 (2)	>100 (3)
Listeria monocytogenes	Ausencia/25g (0)	Presencia/25g (3)		
Salmonella	Ausencia/25g (0)	Presencia/25g (3)		
Staphylococcus coagulasa +	Ausencia/25g (0)	1-50 (1)	51-100 (2)	>100 (3)
Clostridium	Ausencia/25g (0)	Presencia/25g (3)		
Líquido / Salmuera	***Rango concentración (ufc/mL)***			
AMT	$<10^2$ (0)	10^2-10^3 (1)	10^3-10^5 (2)	$>10^5$ (3)
Enterobacterias	<20	2-50 (1)	51-100 (2)	>100 (3)
BAL	$<10^2$ (0)	10^2-10^3 (1)	10^3-10^5 (2)	$>10^5$ (3)
Mohos y levaduras	$<10^2$ (0)	10^2-10^3 (1)	10^3-10^5 (2)	$>10^5$ (3)
Staphylococcus coagulasa +	Ausencia/25mL (0)	1-50 (1)	51-100 (2)	>100 (3)
Clostridium	Ausencia/25mL (0)	Presencia/25 mL (3)		
Agua (Aljibe)	***Rango concentración (ufc/100 mL)***			
AMT	Ausencia (0)	1-50 (1)	51 - 99 (2)	>100 (3)
Coliformes totales	Ausencia/100 mL (0)	Presencia/100 mL (3)		
Clostridium perfringens	Ausencia/100 mL (0)	Presencia/100 mL (3)		
Parámetros físico-químicos	***Rango concentración***			
pH	<4.00 (0)	4.0 - 4.2 (1)	4.3 - 4.5 (2)	>4.6 (3)
acidez libre (g ácido láctico /L)	>0.5 (0)	0.5 - 0.4 (1)	0.3 - 0.2 (2)	<0.1 (3)
Sal (%)	>5.0 (0)	4.9 - 4.5 (1)	4.4 - 4.1 (2)	<4.0 (3)
Aliños (Pimiento, ajo, hierbas)	***Rango concentración (ufc/g)***			
AMT	$<10^3$ (0)	10^3-10^4 (1)	10^4-10^5 (2)	$>10^5$ (3)
Enterobacterias	Ausencia/25g (0)	10-50 (1)	51-100 (2)	>100 (3)
BAL	<100 (0)	10^2-10^4 (1)	10^4-10^5 (2)	$>10^5$ (3)
Mohos y levaduras	<100 (0)	10^2-10^4 (1)	10^4-10^5 (2)	$>10^5$ (3)
Esporulados	Ausencia/25g (0)	10-50 (1)	51-100 (2)	>100 (3)
Staphylococcus coagulasa +	Ausencia/25g (0)	1-50 (1)	51-100 (2)	>100 (3)
Clostridium	Ausencia/25g (0)	Presencia/25g (3)		

Nota: El conocimiento de los expertos del CSIC y la UCO, las condiciones específicas de procesado de cada empresa y la legislación vigentes determinan que parámetros se deben analizar en cada etapa de la cadena de procesado de la empresa.

Posteriormente, la información se agregó a una hoja de cálculo (MS Excel) en la que se implementó el modelo matemático. La Figura 1 muestra una vista parcial de dicha hoja de cálculo con información referente a la fase del proceso, concretamente la Recepción y Almacenamiento en Bombona y su descripción (columnas A y B), el tipo de muestra y los parámetros analizados (columnas D y E). En este ejemplo concreto se muestran resultados correspondientes a dos visitas (columnas F y G). Las columnas H e I muestran las puntuaciones que oscilan entre 0 y 3, ordenados de mayor a menor calidad higiénica.

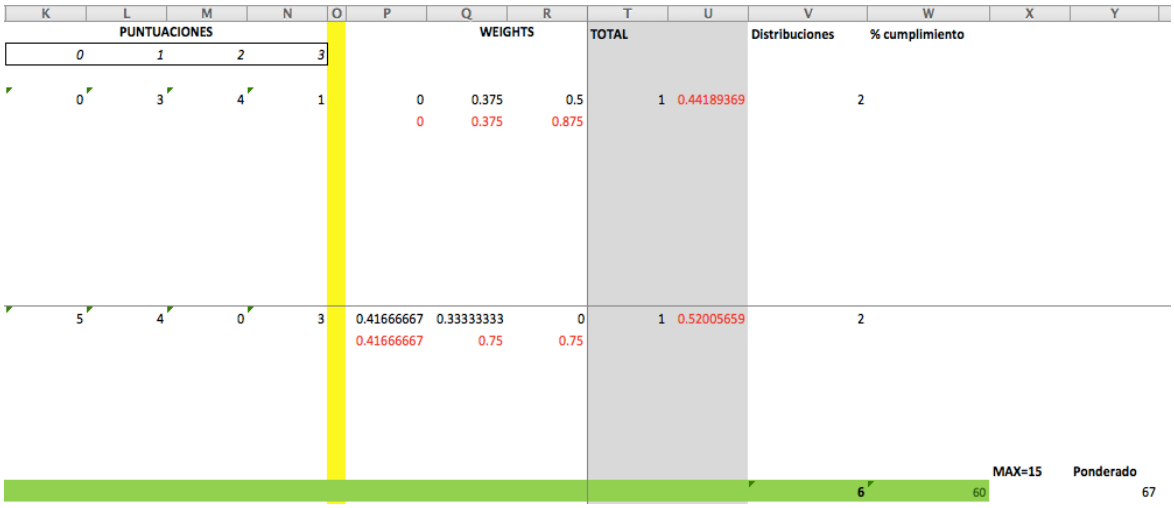
Figura 1: Vista parcial de una hoja de cálculo de MS Excel en la que se muestra la asignación de las puntuaciones de aceituna Aloreña de Málaga en base al cumplimiento de la normativa y recomendaciones aplicables al sector de la aceituna de mesa

FASE NUMERO	DESCRIPCIÓN	TIPO DE MUESTRA	PARÁMETROS	Resultado 1ª visita	Resultado 2ª visita	Punt 1	Punt 2
1	Recepción y almacenamiento en bombona	Ambiente					
		UFC/m3	Aerobios mesófilos	228	>300	2	3
		UFC/m3	Mohos/levaduras	1.95E+02	7.10E+01	2	1
		UFC/m3	Aerobios mesófilos	2.12E+02	2.43E+02	2	2
		UFC/m3	Mohos/levaduras	1.30E+01	1.90E+01	1	1
		UFC/m3	Aerobios mesófilos	1.89E+02	1.67E+02		
		UFC/m3	Mohos/levaduras	3.00E+00	8.00E+00		
		UFC/m3	Aerobios mesófilos	1.10E+02	2.22E+02		
		UFC/m3	Mohos/levaduras	5.40E+01	8.90E+01		
		Salmuera					
		UFC/ml	Aerobios mesófilos	1.83E+06	1.19E+06	3	3
		UFC/ml	Mohos/levaduras	1.91E+05	4.00E+04	3	2
		UFC/ml	Bacterias lácticas	2.09E+06	2.61E+06	3	3
		UFC/ml	Enterobacterias	<20	<20	0	0
		UFC/ml	Aerobios mesófilos	5.04E+05	4.03E+05	3	3
		UFC/ml	Mohos/levaduras	4.25E+03	5.00E+05	2	3
		UFC/ml	Bacterias lácticas	4.54E+03	4.61E+04	2	2
		UFC/ml	Enterobacterias	<20	<20	0	0
		UFC/ml	Aerobios mesófilos	3.00E+01	7.48E+01		
		UFC/ml	Mohos/levaduras	<20	<20		
		UFC/ml	Bacterias lácticas	<20	<20		
		UFC/ml	Enterobacterias	<20	<20		
		UFC/ml	Aerobios mesófilos	6.60E+06	2.02E+06		
		UFC/ml	Mohos/levaduras	3.21E+03	6.60E+04		
		UFC/ml	Bacterias lácticas	8.90E+06	2.31E+06		
		UFC/ml	Enterobacterias	<20	<20		
		Fruto					
		UFC/g	Aerobios mesófilos	1.98E+06	3.87E+06	3	3
		UFC/g	Mohos/levaduras	1.96E+04	1.37E+04	2	2
		UFC/g	Bacterias lácticas	2.13E+06	2.76E+06	3	3

A continuación se realizó un tratamiento estadístico de las puntuaciones que mostró la frecuencia relativa de cada una de las puntuaciones (0-3) por cada fase del proceso (ver Figura 2). Estas frecuencias se ponderaron en base a una distribución estadística discreta, implementada en la columna V. Dicha distribución asocia el valor obtenido (0-3) a su correspondiente probabilidad de aparición. En el ejemplo mostrado en la Figura 2 se observan los resultados correspondientes al análisis de dos muestras. En la primera de ellas, la celda señalada (columna V) devolvería con mayor probabilidad la puntuación 2, ya

que ha sido el valor observado con mayor frecuencia. En la segunda muestra, la celda perteneciente a la columna V tendrá una misma probabilidad para las puntuaciones de 0 y 2. Por último, en la columna W se calcula el porcentaje de cumplimento parcial de cada fase en función de la Ecuación 1.

Figura 2: Vista parcial de una hoja de cálculo MS Excel con la distribución discreta (columna V) para el cálculo probabilístico de la puntuación obtenida para tipo de muestra y fase en aceituna Aloreña de Málaga



Realizando un sumatorio de todas las fases del proceso se obtienen unos porcentajes globales (BASE y PONDERADO) que resultan de la aplicación de las ecuaciones 2 y 3.

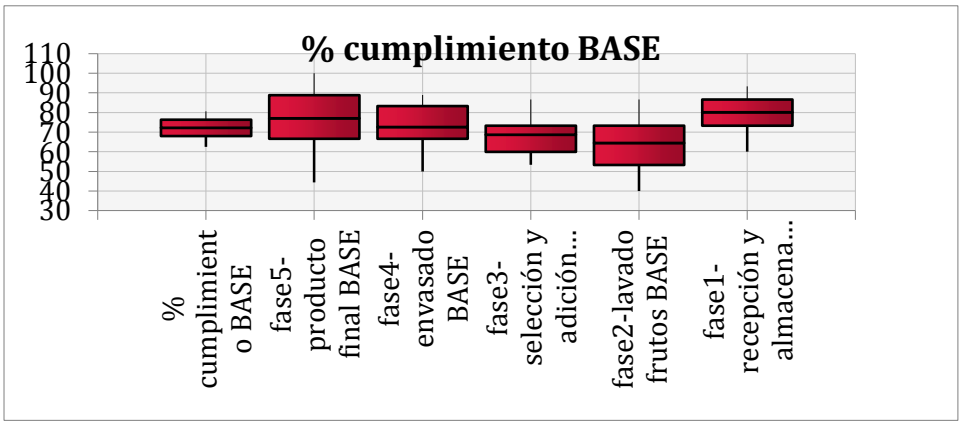
En el caso de utilización de la Ecuación 3 se introducen las ponderaciones correspondientes. Dichas ponderaciones son porcentajes asignados por expertos pertenecientes al sector así como por investigadores del CSIC y de UCO que dan un mayor peso a las distintas fases en relación con el resultado final. En la Tabla 3 se muestra un ejemplo de los porcentajes asignados por 7 expertos a cada fase del proceso. Estos porcentajes consideran la variabilidad entre los distintos expertos mediante una distribución de tipo triangular, que tiene como parámetros el valor mínimo, más probable y máximo de cada fase del proceso.

Tabla 3. Asignación de porcentajes otorgados por un panel de expertos (Instituto de la Grasa, UCO y sector de la Aceituna de Mesa) que representan la importancia relativa de las fases del proceso de elaboración de aceituna de mesa.

FASES	Descripción	Experto1	Experto2	Experto3	Experto4	Experto5	Experto6	Experto7
1	Recepción y almacenamiento en bombona	20	18	15	12	10	5	20
2	Lavado frutos	10	5	7	10	15	11	9
3	Selección y adición aliños	15	20	10	15	25	20	22
4	Envasado	35	30	40	45	30	35	35
5	Producto final envasado	20	27	28	18	20	29	14
TOTAL		100	100	100	100	100	100	100

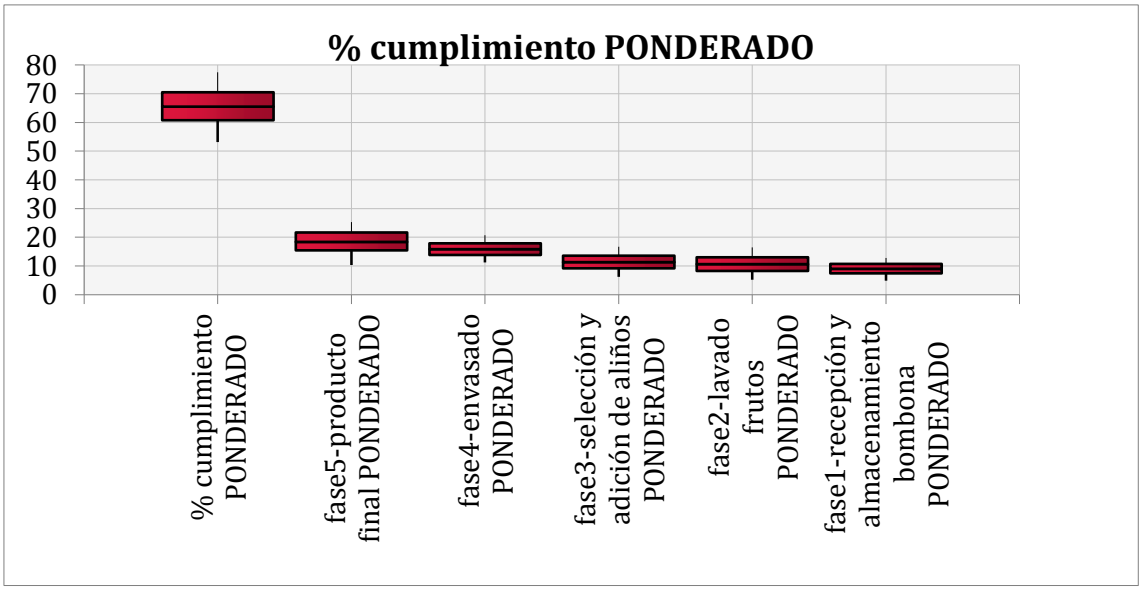
Posteriormente, se obtuvieron representaciones del cumplimiento general y de cada fase, a través de un análisis de riesgo, utilizando el @Risk v7.5 (Palisade Corporation) que lleva a cabo una simulación de MonteCarlo (#10000 iteraciones). Los datos utilizados para las iteraciones se basan en el sumatorio obtenido para cada fase y también de forma global para todas las fases. Ya que el modelo usa distribuciones estadísticas, los resultados finales también se presentan en forma de distribución. El proceso de simulación de MonteCarlo toma de forma aleatoria valores de las distribuciones de inputs para generar una distribución de outputs. La Figura 3 muestra los resultados de dicho análisis sin incluir ponderaciones (usando la Ecuación 2) y referidos al porcentaje de cumplimiento BASE. Estos valores (0-100%) indican la variabilidad del proceso de producción y su influencia sobre la calidad e inocuidad del producto final. En este ejemplo, se comprueba que las fases de lavado de frutos, envasado y análisis del producto final son las que presentan un mayor grado de variabilidad y por tanto, son susceptibles de aplicar medidas correctoras para mejorar y estandarizar la higiene del proceso.

Figura 3: Resultado de la simulación del modelo para la variable % de cumplimiento BASE.



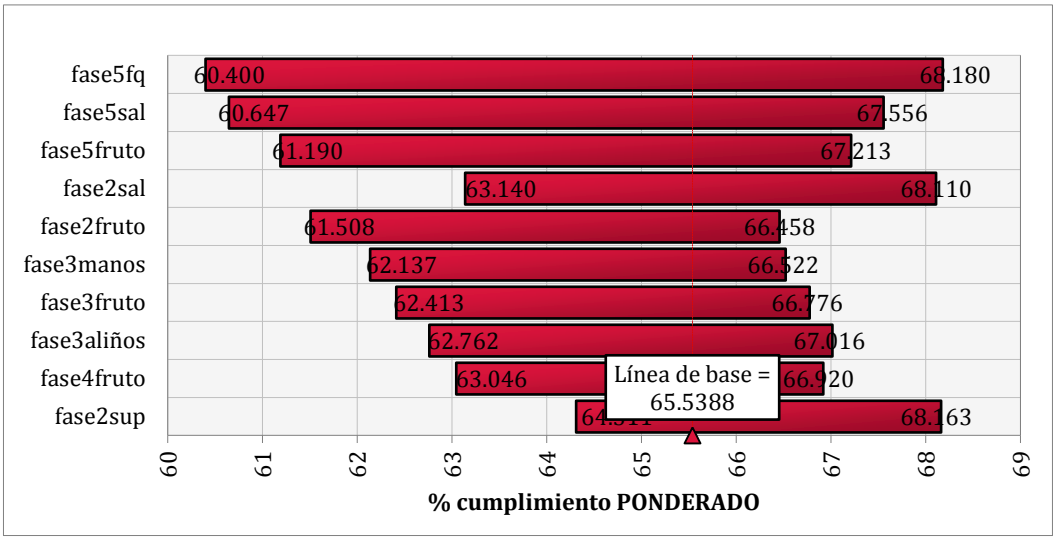
Por su parte, la Figura 4 devuelve los resultados de la aplicación de la Ecuación 3 que contempla la inclusión de ponderación. Se comprueba cómo las fases finales, más cercanas al envasado del producto final contribuyen en mayor medida a la higiene del proceso, ya que la opinión de expertos considera que presentan una mayor relevancia. En cualquier caso, el modelo permite adaptar estas variables de forma que se pueda cuantificar el efecto de posibles medidas correctoras.

Figura 4: Resultado de la simulación del modelo para la variable % de cumplimiento PONDERADO



Finalmente, y utilizando @Risk v7.5 (Palisade Corporation) se realizó un análisis de sensibilidad para determinar el efecto que presentan las diferentes variables de entrada del modelo sobre el output % de cumplimiento ponderado. La Figura 5 muestra cómo las características físico-químicas, así como las condiciones microbiológicas de la salmuera de envasado y producto final (fase 5) determinan en mayor medida el estado higiénico del proceso. En base a esta información, el operario de la industria alimentaria podrá actuar tomando medidas correctoras de forma directa y sencilla, reduciendo costes y optimizando el rendimiento del producto.

Figura 5: Análisis de sensibilidad desarrollado para la variable % de cumplimiento BASE



4. CONCLUSIONES

Las conclusiones más relevantes que se obtienen tras el desarrollo de la presente Tesis Doctoral son las siguientes:

1) El estudio convencional de los principales cambios físico-químicos y microbiológicos que ocurrieron durante el proceso de fermentación y conservación de la aceituna *Aloreña de Málaga* no arrojó gran información. Sin embargo, la aplicación de un modelo matemático ADF programado en R permitió la comparación estadística entre los diferentes tratamiento ensayados, arrojando también información sobre la velocidad y aceleración a la que ocurren los cambios, medias, máximos y mínimos obtenidos con sus correspondientes medidas de error, resultando ser una herramienta de gran utilidad que podrá ser utilizada en posteriores estudios no solo en aceitunas de mesa, sino en la microbiología de los alimentos en general, para la cuantificación y comparación de los cambios físico-químicos y microbiológicos que ocurren durante los procesos. Está conclusión fue obtenida a través del desarrollo de los capítulos 1 y 2.

2) La eliminación de la utilización de cloruro de sodio en el proceso de fermentación y conservación de aceitunas curadas y su sustitución por altas concentraciones de ácido acético para favorecer la conservación de los frutos, no produjo grandes cambios en la evolución de las principales poblaciones microbianas, siendo las levaduras las predominantes durante todo el proceso (sin diferencias estadísticas). Sin embargo, si se observaron cambios significativos estadísticos en la evolución del pH y la acidez libre, sobre todo durante los 3 primeros meses, con una subida paulatina del pH, aunque todos los procesos condujeron finalmente a un alimento seguro (pH $\leq$4,0). No se produjo ningún tipo de alteración de los frutos. Por lo tanto, un proceso industrial donde se eliminara el cloruro de sodio y se sustituyera por una salmuera altamente acidificada podría ser posible, aunque habría que estudiar en detalles que modificaciones organolépticas puede tener sobre el producto final (sabor, color, textura, etc.). Sin embargo, las altas ventajas que tiene reducir los niveles de sodio en la elaboración, tanto desde un punto de vista medioambiental, como para satisfacer los requerimientos de los consumidores, hace necesario implementar procesos de este tipo en las industrias. Esta conclusión fue obtenida a través del desarrollo de los capítulos 1 y 2.

3) La aplicación de técnicas ómicas para el estudio de la evolución de las poblaciones bacterianas a lo largo del proceso de fermentación y conservación de las aceitunas tradicionales *Aloreña de Málaga*, ha permitido la identificación de diferentes especies

bacterianas halófilas, entre ellas *Celerinatantimonas diazotrophica*, no descritas con anterioridad en aceitunas de mesa. Las técnicas clásicas dependientes de cultivo mostraban la ausencia de bacterias y que el proceso fermentativo estaba llevándose a cabo fundamentalmente por levaduras. Sin embargo, la utilización de esta técnica independiente de cultivo, ha revelado la existencia de diferentes grupos bacterianos que se desarrollan durante el proceso tanto en salmuera como en fruto. Queda por determinar que influencia tienen estas nuevas especies sobre la calidad del producto final. También se muestra la necesidad de incluir en los análisis microbiológicos de manera rutinaria un mayor número de medios de cultivo que permitan el estudio de estos grupos microbianos por técnicas dependientes de cultivo. En ningún momento, se ha detectado por las técnicas utilizadas la presencia de especies patógenas durante la fermentación y conservación a niveles poblacionales que pudieran suponer un riesgo para los consumidores, lo cual garantiza una alta seguridad microbiológica para este producto. Esta conclusión fue obtenida a través del desarrollo del capítulo 3.

4) El estudio de metagenómica llevado a cabo sobre la población fúngica (hongos y levaduras), muestra la existencia de una comunidad compleja con la presencia de especies fitopatógenas, saprófitas, alterantes y fermentativas. El perfil de la población se modificó a lo largo del proceso de fermentación y conservación. De este modo, al inicio y en la materia prima, se encuentran especies de los géneros *Penicillium, Cladosporium* y *Malassezia,* mientras que tras 4 meses de fermentación y conservación de las aceitunas las especies predominantes pertenecen a los géneros *Zygotorulaspora* y *Pichia* (con un perfil más fermentativo). Serán necesarios estudios posteriores para profundizar aún más en el papel que estas especies tienen sobre la calidad y seguridad del producto final con el fin de seleccionar aquellas especies que tienen actividades más beneficiosas y desarrollar cultivos iniciadores. Esta conclusión fue obtenida a través del desarrollo del capítulo 4.

5) La aplicación de un escaldado de los frutos a su llegada a la fábrica y previo a su puesta en salmuera, fue beneficioso para el desarrollo de la fermentación láctica, retención del color verde de los frutos y mayor estabilidad de los envasados, especialmente para el proceso de elaboración de aceitunas *Aloreña de Málaga* tradicionales. Este procedimiento, aparentemente fácil de implementar e introducir en la cadena de producción de las industrias, supondría una gran mejora en la calidad de los frutos (mayor color verde), estabilidad y

seguridad de los envases (se consigue un pH más bajo y mayor acidez libre). Asimismo, este tratamiento no produce ningún tipo de efecto adverso sobre las características organolépticas de las aceitunas (textura, sabor, etc.) o valoración de los envasados. Esta conclusión fue obtenida a través del desarrollo del capítulo 5.

6) Las visitas realizadas a las empresas del sector para la toma de muestras físico-químicas y microbiológicas además del estudio de sus sistemas de APPCC, muestran que existe una falta de estandarización en los procesos, que hay etapas potencialmente mejorables (lavado y aliñado) y que el grado de cumplimiento higiénico-sanitario aumenta considerablemente en las últimas etapas del proceso de elaboración solo prestando un poco más de atención a las etapas anteriormente indicadas, ya que son fuente, de gran variabilidad microbiológica al final del proceso. Esta conclusión fue obtenida a través del desarrollo del capítulo 6.

7) No se detectó en ninguna muestra de envasado la presencia de géneros patógenos que pudieran suponer un riesgo para los consumidores, lo cual garantiza una vez más la seguridad del producto final. Con toda la información obtenida, se ha diseñado un protocolo y procedimiento científico basado en un sistema de ponderación y análisis probabilístico del riesgo para la gestión de la calidad y seguridad alimentaria en las empresas del sector, que ayude a determinar qué etapas tienen potencial de ser mejoradas y cómo la introducción de medidas correctoras (ozonización, irradiación o escaldado de aliños, compartimentalización de instalaciones, etc.), pueden afectar muy positivamente al proceso. Este enfoque, aunque ha sido diseñado teniendo en cuenta los procedimientos que se llevan a cabo en la industria de la *Aloreña de Málaga*, está siendo extrapolado a otros tipos de elaboraciones de aceitunas de mesa y también a otros sectores de la alimentación junto con la implementación en herramientas informáticas que faciliten su manejo. Esta conclusión fue obtenida a través del desarrollo del capítulo 6.

CURRICULUM VITAE

Licenciado en Ciencias Biológicas por la Universidad de Málaga (1997), Técnico Superior en Prevención de Riesgos Laborales (CEM – 2007), Máster en Dirección de Empresas de Economía Social (EAES – 2010/11) y Máster en Agroalimentación (Universidad de Córdoba – 2014). Antes de la realización de esta tesis doctoral trabajó en el laboratorio de Bioquímica y Biología Molecular de la Universidad de Málaga (1997-1998) donde realizó 2 publicaciones científicas sobre la regulación de la actividad de células tumorales.

Amplia formación en la gestión empresarial, societaria, fiscal, contable y el ámbito de la calidad y seguridad alimentaria para empresas del sector agroindustrial, fundamentalmente relacionado con el mundo de la aceituna (almazaras y transformación de aceitunas de mesa). Conoce bien el sector oleícola, ya que ha sido vicepresidente y, posteriormente, presidente, de la Cooperativa El Molino de Guaro, SCA (2008-2014 – 2014-actualidad) y presidente desde su fundación de la SCA Sierra de las Nieves – Grupo *Aloreña* de 2º Grado (2011-2017), siendo la entidad cooperativa que mayor volumen de comercialización de aceituna con DOP *Aloreña de Málaga* gestiona. Actualmente es responsable técnico de los Centros de Apoyo y Desarrollo al Emprendedor (CADEs) de Guaro y Alozaina (Málaga), centros dependientes de Andalucía Emprende, Fundación Pública Andaluza (Consejería de Economía y Conocimiento, Junta de Andalucía). Posee una dilatada trayectoria de más de 15 años en el asesoramiento, formación, difusión y dinamización de la cultura emprendedora tanto para nuevos empresarios como para empresas ya consolidadas, sobre todo, en el ámbito de la agroindustria y el sector primario.

Socio y fundador de la Empresa de Base Tecnológica *Technological Applications for Improvement of the Quality and Safety in FOODs*, que licencia tecnología del CSIC y la UCO obtenida en la presente tesis doctoral (secreto industrial 3731/2016).